Brain serotonin throughout development-

for better and for worse

Der Effekt von Serotonin im sich entwickelnden Gehirn-in guten wie in schlechten Tagen

DISSERTATION

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by

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As you know, in most areas of science, there are long periods of beginning before we really make progress.

- Eric Kandel

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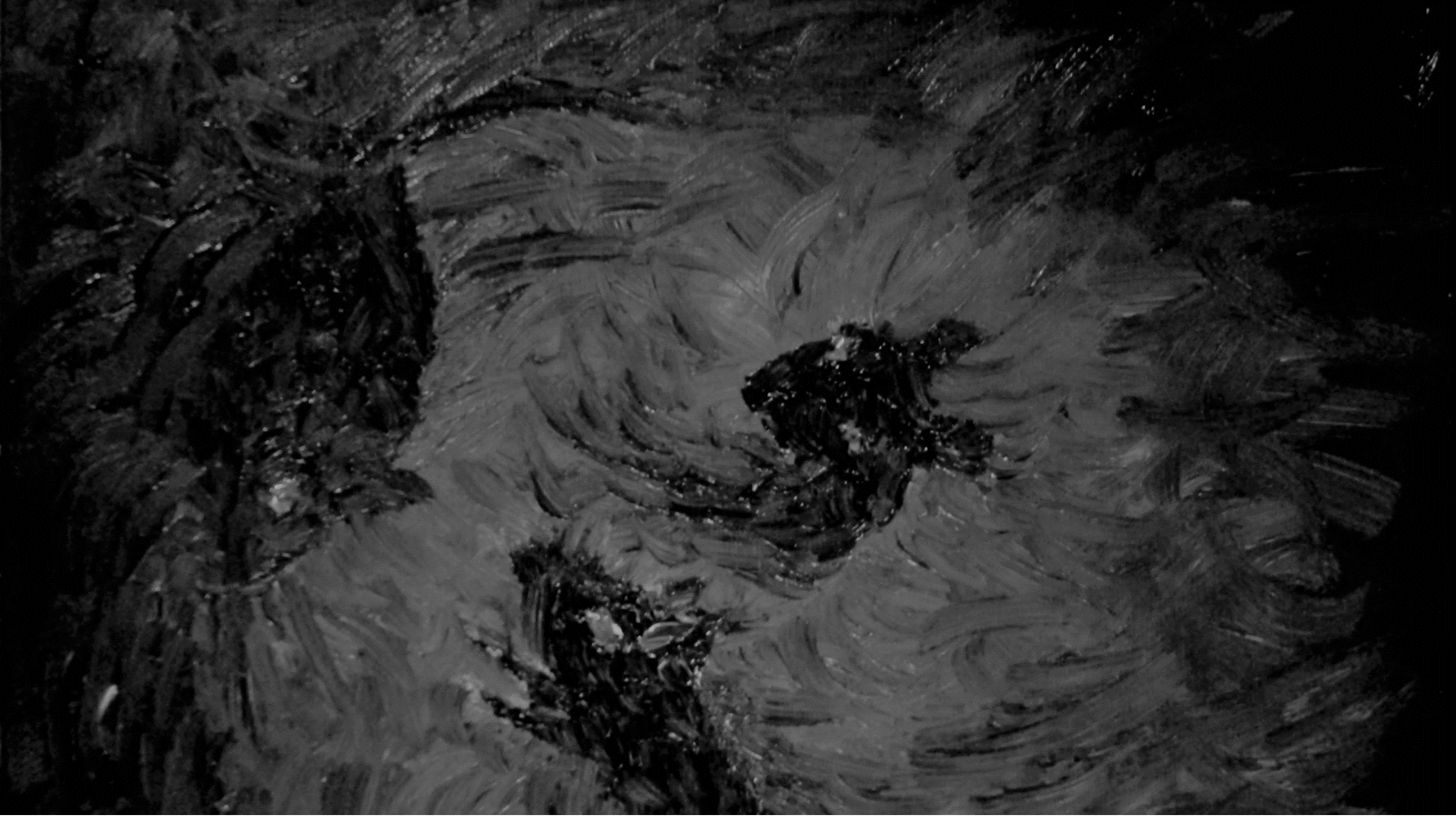

General introduction

Developmental stress exposure often has negative effects on neural development. These effects carry over into later life and compromise mental health. Amongst common developmental stressors are neglect and physical as well as sexual abuse, which have been identified as non-specific risk factors for mood and anxiety disorders, including major depression (MD), generalised anxiety disorder (GAD) or posttraumatic stress disorder (PTSD) (Heim *et al.*, 2010). Nevertheless, a substantial number of individuals displays remarkable stress coping abilities, proving to be unaffected by adversity, experienced during early life/childhood. This dichotomy is influenced by many factors, such as social support by parents or peers, but also the period of exposure, and genetic predisposition (Heim *et al.*, 2010). Both genetic predisposition and environment are of critical importance throughout development to determine mental health (Homberg and van den Hove, 2012). Disturbances, of either the genetic make-up or the environment may lead to altered developmental trajectories and, consequently, altered susceptibilities to subsequent challenges. At the molecular level, changes that are caused by genetic polymorphisms or environmental adversities, are transduced by epigenetic mechanisms (Dudley *et al.*, 2011). Epigenetics comprises a multitude of modifications that occur at the DNA strand itself, or at the nucleosome-forming histone molecules, and define the structural state of chromatin. To date, two forms of DNA-related modifications, i.e. methylation and hydroxymethylation of the cytosine base (Guo *et al.*, 2011), and numerous histone tail modifications have been identified (Kouzarides, 2007). First studies towards an understanding of the interactive and mediatory actions of epigenetic regulation found proof for the involvement of the serotonin (5-hydroxytryptamine, 5-HT) system in epigenetic mechanisms of early adversity, affecting, for example, hypothalamic-pituitary-adrenal (HPA) axis reactivity, which is one of the major determinants of susceptibility (Hellstrom *et al.*, 2012).

In the brain, 5-HT is synthesised in a two-step reaction, driven by the rate limiting enzyme tryptophan hydroxylase 2 (TPH2), which is expressed only in a small group of neurons forming the raphe nuclei (Gutknecht *et al.*, 2009). Projections, emerging from the raphe nuclei, reach the brain stem and various forebrain structures, allowing modulatory actions of brain 5-HT in nearly every brain region. The physiological effects of 5-HT are mediated by several 5-HT receptors that are located at the pre- and post-synapse. Subsequent to receptor binding, the presynaptic 5-HT transporter (SERT, 5-HTT) terminates the effects of 5-HT by transporting 5-HT back into the cell. Next to associations with basic physiological functions, 5-HT modulates the activity of other neuronal networks, and shapes cognition and complex emotional behaviours during development (Gaspar, Cases and Maroteaux, 2003; Wirth, Holst and Ponimaskin, 2017). Genetic variants of the 5-HT system are associated with an increased risk for antisocial behaviour, anxiety and depressive traits (Lesch *et al.*, 1996; Caspi *et al.*, 2002, 2003) as well as altered reactivity of stress-relevant brain regions (Canli *et al.*, 2005; Canli and Lesch, 2007). Most mouse models, in which components of the 5-HT system have been manipulated, show mild to severe behavioural changes, often related to altered stress reactivity. Studies, investigating models of brain 5-HT deficiency, using mouse lines depleted of TPH2, found a variety of behavioural and physiological changes, such as increased aggression and alterations in anxiety-related behaviours (Mosienko *et al.*, 2014; Gutknecht *et al.*, 2015). Furthermore, 5-HT deficiency affects stress reactivity and HPA axis functioning (Gutknecht *et al.*, 2015). Studies, investigating 5-HTT deficiency in mice,

which is leading to altered 5-HT homeostasis and increased 5-HT synthesis (Kim *et al.*, 2005), found alterations in social and anxiety-related behaviours as well as in baseline corticosterone and HPA axis responsiveness (Murphy and Lesch, 2008). Evidently, the consequences of a dysregulated 5-HT system are manifold and not necessarily related to a general lack of 5-HT but can be the consequence of an altered homeostasis. One possible explanation for the wide variety of consequences is that 5-HT exerts several layers of effects, concerted by the interplay of specific 5-HT receptors (Gaspar, Cases and Maroteaux, 2003; Wirth, Holst and Ponimaskin, 2017). Therefore, the effects of a dysregulated 5-HT system can prompt a multitude of small, individual effects on different brain circuitries, dependent on the period in development, the type of neuron and the affected component of the 5-HT system (Gaspar, Cases and Maroteaux, 2003; Lupien *et al.*, 2009; Migliarini *et al.*, 2013).

5-HT can be detected early in development (Golden, 1973) and modulates basic, morphogenetic processes including cell proliferation, migration and circuit wiring (Lipton and Kater, 1989; Lauder, 1993; Levitt *et al.*, 1997; Azmitia, 2001; Vitalis and Parnavelas, 2003). 5-HT of placental and maternal origin is suggested to be critically involved in these processes during developmental stages that precede the appearance of foetal 5-HT neurons (Côté and Fligny, 2007; Bonnin and Levitt, 2011). 5-HT deficiency in the foetus affects only the fine-tuning of 5-HT circuitries, resulting in altered innervation in forebrain regions, including the suprachiasmatic and thalamic paraventricular nuclei, the nucleus accumbens and the hippocampus (Migliarini *et al.*, 2013). This observation suggests that, the fundamental wiring of the brain is initiated prenatally, includes processes related to the development of the 5-HT system itself, and is supported by the placental/maternal 5-HT system, while the final fine-tuning of the circuitries is regulated by 5-HT signalling during the early postnatal period (reviewed in (Gaspar, Cases and Maroteaux, 2003; Deneris and Gaspar, 2017)).

Interestingly, experiments that were modelling early-life adversity have been associated with molecular changes, which, at least in part, were related to the 5-HT system, and behavioural changes that were comparable to the changes observed in genetic models of 5-HT system dysfunction (e.g. (van den Hove *et al.*, 2014; Wong *et al.*, 2015; Hiroi *et al.*, 2016)). Such adversity-induced changes in the 5-HT system were associated with epigenetic changes at 5-HT-related genes (Kinnally, Capitanio and Leibel, 2010; Márquez *et al.*, 2013). As previously mentioned, functioning of the 5-HT system was also related to processes, involved in the epigenetic reprogramming of HPA axis reactivity, in the context of early-life experiences, such as the quality of maternal care (Hellstrom *et al.*, 2012), altogether, suggesting a possible route of effect of early experiences via the 5-HT system, with the involvement of epigenetic mechanisms. This would make the molecular consequences of early adversity prone to genetically-induced changes of 5-HT functioning. In line with this notion, several groups showed that mice with reduced brain 5-HT levels display altered susceptibility to early stress, dependent on the type of stress, further experiences throughout life, and type of behavioural test performed (Sachs *et al.*, 2013, 2015; Wong *et al.*, 2015). Studies in our group found that mice, deficient for brain 5-HT, showed an altered HPA axis activity and were resilient to chronic mild stress-induced hyperlocomotion, while the same stressor intensified conditioned fear responses in a gene-by-environment interaction (GxE)-dependent manner (Gutknecht *et al.*, 2015). Furthermore, we were able to associate differences in gene expression, DNA methylation, behavioural coping and HPA axis activity measures with the

interaction of 5-HTT availability and prenatal stress (PS) (van den Hove *et al.*, 2011; Jakob *et al.*, 2014; Schraut *et al.*, 2014).

In conclusion, extensive research suggests a crucial role of 5-HT signalling in the modulation of effects, induced by early adversity. Genetic variation of components of the 5-HT system might, thus, be permissive or attenuative for the effects of early adversity (Homberg and van den Hove, 2012).

Overview of the thesis

The research presented in this thesis is divided into three parts. **Chapter 2** comprises a review, providing an overview of the current knowledge in epigenetic programming by early-life stress, in the context of 5-HT system modifications, with a focus on aggression-related pathology. **Chapters 3** and **4** report findings of behavioural and molecular screenings, following GxE interactions of diverse components of the 5-HT system with adversity during relevant, developmental periods. Finally, in **chapters 5** and **6**, we used a more targeted approach. While in **chapter 5** brain circuitries, involved in anxiety-related behaviours, were investigated, one candidate gene, discovered in a previous study (**supplementary Chapter** (Schraut *et al.*, 2014)), was followed up on, in **chapter 6**, in an attempt to validate findings, obtained using animal research, in the relevant context of early adversity in humans.

While the current chapter only briefly lays out the complex, reciprocal actions of 5-HT and early adversity, **chapter 2** dives into a detailed elaboration of the findings in numerous 5-HT system-modified mouse models and models of early adversity of the last decades. In this review, we give a short overview of aggression-related physiology. Subsequently, the prominent role of 5-HT in the manifestation of aggression, in the context of early adversity, is laid out. Moreover, this chapter highlights the crucial role epigenetic programming plays in such processes and points out a need of more integrative studies, to allow the determination of distinct developmental mechanisms.

In **chapter 3**, this reciprocal relationship of 5-HT and early adversity as well as their behavioural consequences was investigated in a model of maternal separation (MS) in mice of a *Tph2*-deficient mouse line (Tph2xMS). The focus of this study was on behaviours of emotional dysregulation, such as in exaggerated anxiety and aggression. Findings showed a general effect of full *Tph2* deficiency, profoundly affecting behaviours as well as the molecular make-up of the brain, in such a manner that promoted an overall more activity-oriented phenotype, which might be indicative of MS-induced effects on behaviour. Furthermore, we suggest that *Tph2* deficiency led to alterations in epigenetic processes, related to early adversity.

Differential susceptibility to early adversity is an important cue towards mental well-being and a wealth of research suggested 5-HT system-dependent mechanisms to be involved. The *5-HTT* gene-linked polymorphic region (*5-HTTLPR*), in particular, caught the interest of research, investigating GxE interactions in humans. Therefore, **chapter 4** employed a mouse model of *5-Htt* deficiency, in order to investigate its association with differential susceptibility to early adversity and to identify potential mechanisms involved in programming such distinct phenotypic outcomes. To this end, *5-Htt*-deficient mice were exposed to stress throughout the last phase of gestation. This resulted in various behavioural manifestations, which were found to be expressed, dependent on the *5-Htt* genotype.

Molecular analyses supported this notion, suggesting genotype-dependent programming of behaviour and their molecular correlates by developmental stress.

To further investigate the morpho-functional consequences of early adversity, the final two chapters employed immunohistological methods. In **chapter 5**, we investigated the effects of an interaction of 5-HT deficiency and early adversity on neural activity in anxiety-related brain regions. To meet this end, mice of the previously described Tph2xMS model were behaviourally tested in the open-field and dark-light box test during adulthood. Subsequently, their brains were used for the immunohistological investigation of neural activation by c-Fos immunohistological staining. In this study, results suggest that, dependent on the availability of brain 5-HT and environmental context, MS promoted active responses to aversive stimuli, which seemed to be related to distinct neural activation in several subnuclei of the amygdala, the paraventricular nucleus and the periaqueductal grey.

Chapter 6 finally focussed on the validation of a candidate gene, i.e. myelin basic protein (*Mbp*), that previously was shown to be affected by the interaction of 5-HTT deficiency and PS, both at the level of DNA methylation and mRNA expression in the hippocampus of adult, female mice (**supplementary chapter** (Schraut *et al.*, 2014)). In this chapter, we aimed to replicate the effect of prenatal environment on the *Mbp* methylation in mouse brain and human blood. Furthermore, we investigated the origin of the observed alteration in *Mbp* expression and employed several histological approaches to this end. Results of this investigation suggest 5-HT-sepcific, tissue- and species-independent priming of the *Mbp* gene locus by maternal stress experience during pregnancy, in particular in female offspring.

Taken together, all studies, conducted during the course of this PhD project, point to the important and active role 5-HT plays in the modulation of effects, induced by early adversity and transduced by epigenetic mechanisms. Moreover, first steps towards unravelling the down-stream effectors of such programming have been undertaken, creating a foundation for future research that will finally lead to interventional strategies e.g. on the behavioural level, or guiding more invasive therapies such as deep brain stimulation, fitted for the individual patient, based on their molecular make-up and life-history.

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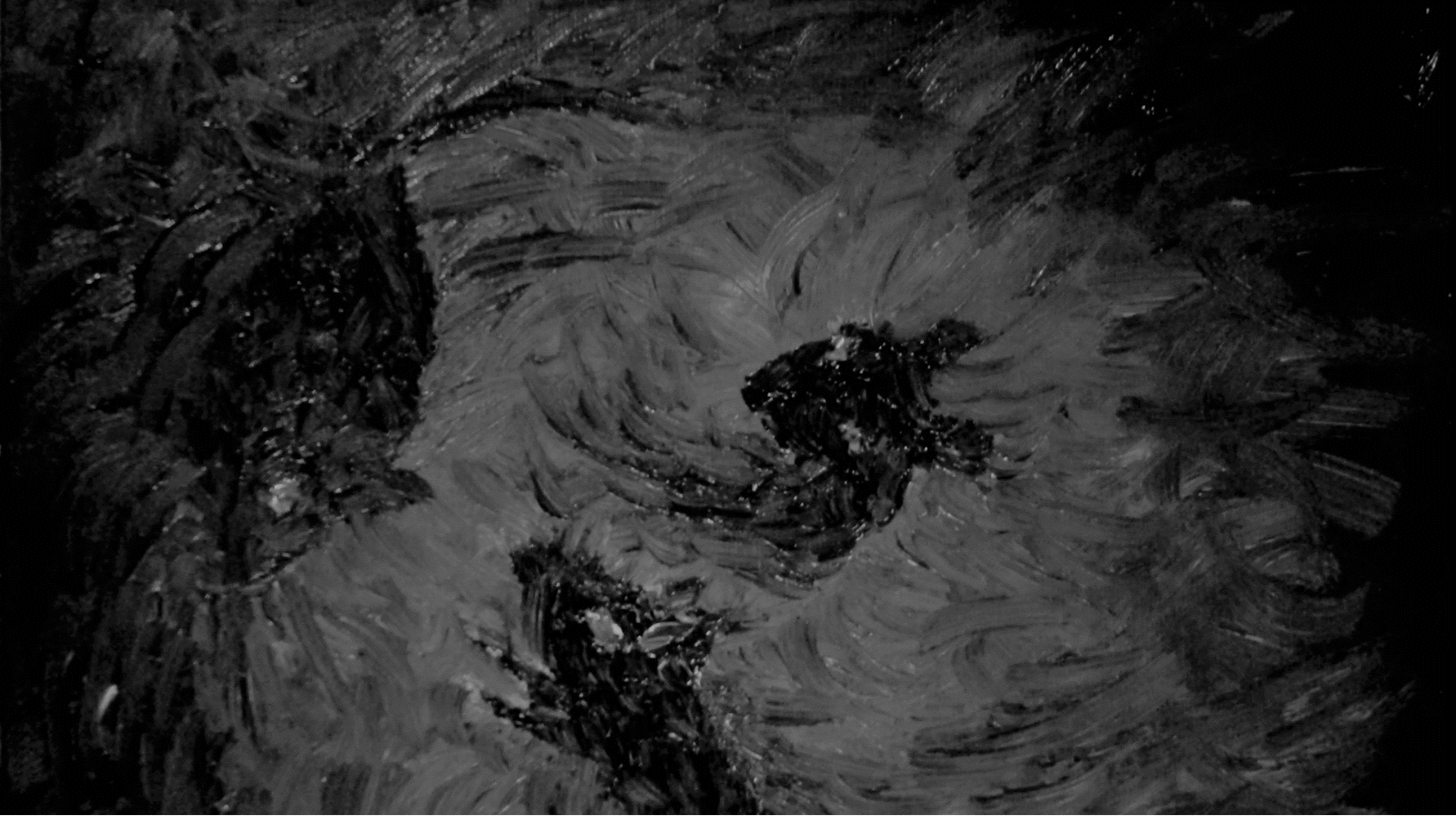
Chapter I
General introduction



Gene-by-environment interaction in aggression: the role of developmental, epigenetic programming

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Abstract

In today's society violence is a major burden touching upon public and private life. The aetiology of violent manifestations is as heterogeneous and manifold as the manifestations themselves, rendering the search for unitary causes futile. In this review, we will discuss the molecular changes, induced and affected by early-life adversity, which has been suggested to promote aggression-related psychopathology. However, even with the current detailed sub-classification of aggression and a large body of work focusing on underlying mechanisms, the field is nowhere near to understand all facets of aggression. It seems though, that in case of abnormal aggression-related behaviours, the serotonin (5-hydroxytryptamine, 5-HT) system serves as a distinctive regulator for a subset of aggressive traits. In detail, we suggest that exaggerated, sociopathy-like aggression is most likely induced by early adversity via changes in 5-HT signalling that were brought about by and are also causally involved in, inducing epigenetic modifications. Finally, we point out the importance of more integrative working models, regarding underlying mechanisms of early adversity. Future work should focus more on unravelling gene accessibility and consequent transcriptional activity by examining chromatin as regulatory machinery that integrates multiple layers of environmental input over time.

Introduction

In today's society, violence has been identified as a major burden. It affects public and private life and no country or community remains untouched by the events of violence that occur daily. Statistics of the world health organisation (WHO) report over 1.6 million deaths per year attributable to violence, including suicide, homicide and war (Penden, McGee and Krug, 2000). Next to immediate death, violence is also the cause for impaired health in a notably higher number of victims causing immense economic costs for society (Penden, McGee and Krug, 2000; World Health Organization, 2002). The aetiology of violent manifestations is as heterogeneous and manifold as the manifestations themselves. Next to genetic predisposition, environmental adversity has been identified as a key modulatory factor in the development of mental disturbances, including abnormal, aggression-related behavioural traits (Lesch and Merschdorf, 2000). Both genetic and environmental factors are of critical importance throughout development to determine mental health. Subtle modifications of either may lead to altered developmental trajectories and, consequently, altered behaviour. At the molecular level, modifications in development are transduced by epigenetic mechanisms (Dudley *et al.*, 2011). Epigenetic regulation comprises multiple layers that define the structural state of chromatin and are mediated by modifications at the DNA strand itself, or at the nucleosome forming histone molecules. First studies towards an understanding of such interactive and mediatory actions of early adversity and epigenetic regulation in humans found proof for an association of epigenetic mechanisms with changes in behaviour (Tremblay, 2008; Provençal *et al.*, 2013, 2014). However, clinical studies have inevitable confounders such as smoking, or age of mother at birth that obstruct determining whether a particular genotype or event is causal for a particular behavioural outcome. The use of animal models has, thus, become vital for studying the relationship between environment and genome, allowing for a more stringent control of variables, including the timing and duration of exposure to stress and most other environmental variables as well as life circumstances such as successful mating, food deprivation or defeat experiences, which can lead to territorial, defensive or frustration related aggressive behaviour (e.g. (Takahashi *et al.*, 2012)). Regardless of the species, developmental stress typically has modulatory effects on neural development that carry over into later life and affect behaviour (Sanchez, Ladd and Plotsky, 2001; Weinstock, 2008). Similarly, genetic predispositions can be translated across species (Lesch and Merschdorf, 2000). Therefore, comparative approaches might help identify behavioural outcomes and their underlying mechanisms that co-vary across species.

In the following review, we will describe diverse animal models of aggression, as well as various molecular approaches, that were undertaken to date, in order to unravel the aetiology of abnormal, aggressive behaviour, with a focus on recent advances in the field of epigenetics.

Animal models of aggression

Throughout evolution, aggression has been identified as one of the most important forms of social communication. Its numerous functions range from the establishment of social hierarchies to the acquisition of e.g. food or mating partners. It is therefore important to distinguish these forms of aggression from more deviant manifestations (Miczek, De Boer and Haller, 2013). To get a better understanding of when aggression is abnormal and to identify what kind of abnormal aggression is

displayed, we will first take a closer look at the underlying framework of aggressive encounters and the associated molecular processes.

The frame-work of aggressive encounters

Across a wide variety of species, breeding males defend their territories and attempt to dominate or, alternatively, exclude other males (Blanchard and Blanchard, 2003). In mice, prevalent forms of aggression occur in situations of social conflict, when males defend a territory against intruders, i.e. intermale aggression (Miczek *et al.*, 2001). Like in rodents, primate populations inhabit and defend territories, but while in mice usually one male occupies one territory, primates display more complex social dynamics (Willems, Hellriegel and van Schaik, 2013). In macaques, the two major identifiable forms of aggression are described as defensive and offensive aggression (Kalin, 1999).

The trigger of territorial aggressive behaviour, as described above has been studied intensively in rodents. There, aggression-related motor and neuroendocrine responses are elicited via the concerted actions of the vomeronasal organ (VNO) and the main olfactory epithelium (MOE) (Chamero *et al.*, 2007). In the mouse VNO, two populations of sensory neurons have been identified, either of which expressing components of different G-protein signalling cascades, and resulting in phospholipase C activation (Chamero *et al.*, 2007, 2011; Ben-Shaul *et al.*, 2010), while MOE signalling is mediated via the activation of cyclic-nucleotides (Mandiyan, Coats and Shah, 2005). Animals lacking olfactory signalling in either VNO or MOE display notably decreased levels of aggressive behaviour (Stowers *et al.*, 2002; Mandiyan, Coats and Shah, 2005). Following an activation of MOE or VNO, an excitatory signal goes through the main olfactory (MOB) and accessory olfactory bulb (AOB), respectively, towards multiple regions of the forebrain and the brainstem. Complex signals were shown to be integrated in particular in the hypothalamus (Stowers, Cameron and Keller, 2013), which seems to be an anatomical hub for appraisal and consequential behaviours (Kruk, 2014). Next to the hypothalamus, amygdalar, septo-hippocampal, and fronto-cortical (FC) regions as well as the dorsal raphe (DR) have been identified as down-stream targets of VNO and MOE activation (Anderson, 2016). These brain regions in turn communicate towards the periaqueductal grey (PAG) and hypothalamus, the pituitary gland and sympathetic ganglia, generating a behavioural output as schematically depicted in figure 1 (Veenema and Neumann, 2007; Stowers, Cameron and Keller, 2013; Bergan, 2015; de Boer *et al.*, 2015).

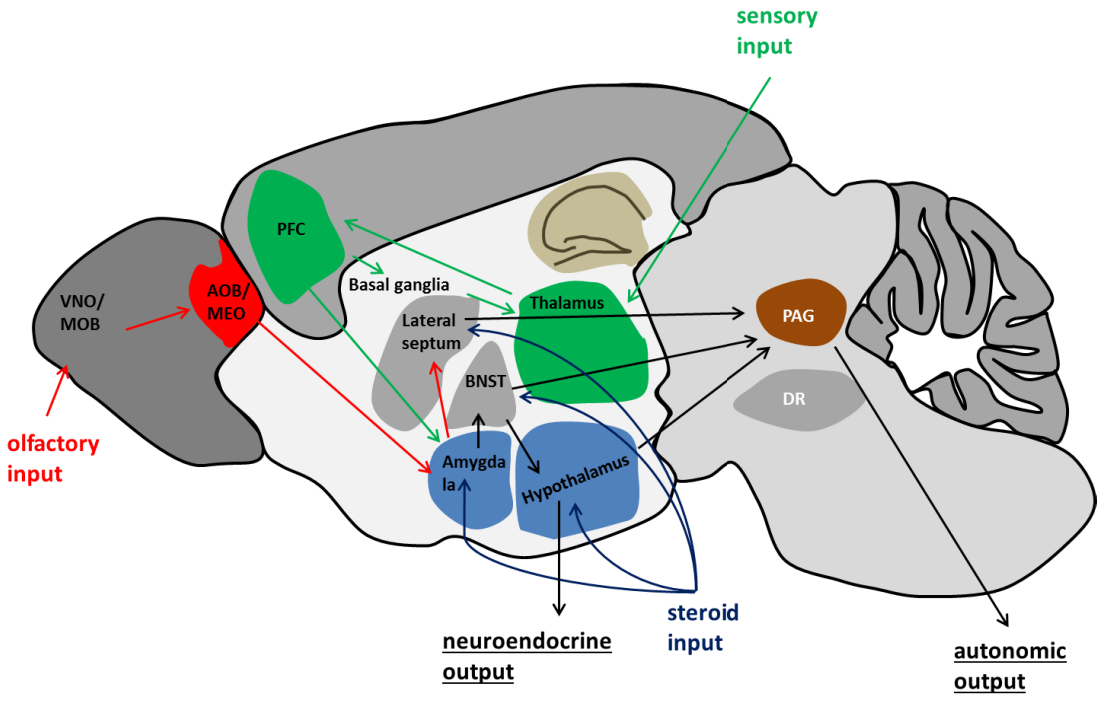

Figure 1 Input and processing of environmental signals that evoke aggression-related, behavioural output. The environmental input is ternary: olfactory signals enter via the vomeronasal organ (VNO) and the main olfactory bulb (MOB) and are processed through the accessory olfactory bulb (AOB) and the main olfactory epithelium (MOE) that project to the amygdala and via this route to the limbic system, where they evoke an autonomic output by activating cells of the periaqueductal grey (PAG). Gonadal steroids impact on the hypothalamus, bed nucleus of the stria terminalis (BNST), amygdala and the lateral septum and evoke or enforce a neuroendocrine output. Further sensory input is processed via the thalamus and the prefrontal cortex (PFC).

A recent imaging study in humans found that aggression-related chemo-signals modulate limbic system activation (Mutic *et al.*, 2017), suggesting that the pathway described in rodents might be relevant, at least in part, in the manifestation of human aggression and reaction to aggressive encounters.

At the molecular level, various neuropeptides and hormones are crucially involved in the manifestation of aggression. In particular, neuropeptides such as arginine vasopressin (AVP) and oxytocin (OXT) have been associated with aggressive behaviour (Veenema, Bredewold and Neumann, 2007). A finding that is not unexpected, given that AVP and the closely related OXT represent the two most investigated “social” peptides. Receptors for both of these neuropeptides, namely the AVP receptors 1a and 1b (AVPR1a, AVPR1b) as well as the OXT receptor (OXTR), are expressed in all brain regions relevant for aggression (Stoop, 2012). AVPR1b knockout mice display mildly impaired social recognition as well as notably reduced aggression (Wersinger *et al.*, 2002). Further interesting findings in this model were a blunted hypothalamo-pituitary-adrenal (HPA) axis activity following stress exposure (Lolait, L Q Stewart, *et al.*, 2007; Lolait, Lesley Q Stewart, *et al.*, 2007). AVPR1a knockout mice showed impaired social recognition, as well as decreased anxiety-like behaviour (Bielsky *et al.*, 2004). Both findings suggest a possible role of these neuropeptides in social appraisal and thus the manifestation of adequate social behaviour.

Furthermore, steroid hormones such as testosterone promote aggressive behaviours (Oortmerssen, Dijk and Schuurman, 1987) via specific receptors (Ogawa and Lubahn, 1997; Ogawa *et al.*, 1998; Marie-Luce *et al.*, 2013). The androgen receptor (AR) and the estrogen receptor α (ESR1) have both

been linked to aggressive behaviour. Furthermore, their functioning was associated with the expression of AVP (Scordalakes and Rissman, 2004). They showed that, dependent on the availability of the ESR1 and the AR, specific brain regions had increased or decreased AVP levels. Interestingly, while Marie-Luce and colleagues (Marie-Luce *et al.*, 2013) found that AR knockout mice display reduced aggressive features, Scordalakes and co-workers (Scordalakes and Rissman, 2004) showed decreased aggression only in ESR1 knockout animals. One possible explanation for this discrepancy in behavioural display is the nature of the employed knockout strains (He *et al.*, 1994) or the experimental set-up (Scordalakes and Rissman, 2004). The role of ESR1 was furthermore supported by experiments employing optogenetics. Optogenetic activation of hypothalamic neurons expressing the ESR1, was sufficient to initiate attacks (Lee *et al.*, 2014). Furthermore, the continuous activation of these neurons was necessary to maintain the antagonistic behaviour.

Lastly, stress hormones represent another important molecular correlate to be taken into account, when discussing the underlying mechanisms of aggression. It is important to note that an aggressive interaction is a source of stress for all parties involved. Their HPA axis is already activated in anticipation of an encounter (Haller, Barna and Baranyi, 1995) and while the winner shows a rapid decrease in glucocorticoid (GC) levels, the loser maintains elevated levels for a longer period of time (Bronson and Eleftheriou, 1965a, 1965b; Schuurman, 1980; Haller, Do and Makara, 1996; Haller *et al.*, 1998; Haller, 2014a, 2014b). These findings suggest an ambiguous role of GCs in aggressive behaviour, which is based on the different nature of their chronic, most likely genomic versus acute, non-genomic effects as discussed by Haller et al. (2014b; 2014a). A series of studies, subjecting animals to acute GC treatment, found that GCs increase aggressiveness in several rodent species (Brain and Haug, 1992; Haller, Albert and Makara, 1997; Kruk *et al.*, 2004; Mikics, Kruk and Haller, 2004). Similar effects were found when GCs were infused into the cerebral ventricles (Mikics, Kruk and Haller, 2004). However, this GC-dependent increase in aggressive behaviour was only found in the context of social challenge, whereas neutral challenges resulted in increased risk-assessment behaviour, instead (Mikics, Kruk and Haller, 2004; Mikics *et al.*, 2005; Mikics, Barsy and Haller, 2007). In contrast, chronic corticosterone exposure increased submissive behaviour. However, as with the acute effects, the observed effects of chronic corticosterone were context dependent and, in naïve mice, only apparent at very high levels, while lower doses were sufficient when administered following defeat. Thus, prior defeat and corticosterone seem to achieve a reciprocal facilitating effect for submissive behaviour (Leshner *et al.*, 1980).

Deviant aggression

As previously mentioned, aggression is an evolutionary adaption. It allows the establishment of social hierarchies, which in turn prevent the need for continued aggression. As aggression is highly cost-intensive, evolution has promoted the use of ritualised behaviours that allow an estimate of the opponent's strength without suffering the consequences of a costly encounter (Natarajan and Caramaschi, 2010). To operate these social interactions successfully, animals must be receptive to sensory feedback such as submissive postures and withdrawal. Following ritualised "threat displays", without either of the opponents retreating, a phase of intense fighting with biting, kicking, and striking

can be observed. At any point, withdrawal is possible and will unconditionally stop the dominating opponent attacking as well as induce post-conflict affiliative behaviour.

Abnormal aggression can manifest as a multitude of deviant behaviours. Studies focussing on the typing of such behaviours have been undertaken in humans and non-human primates (Kalin, 1999) and two distinct subtypes of human aggression, an “impulsive–reactive–hostile–affective” subtype and a “controlled–proactive–instrumental–predatory” subtype were described (Vitiello and Stoff, 1997). These subtypes are also referred to as sociopathy and psychopathy respectively (Yildirim and Derksen, 2013). The first evidence, suggesting that abnormal aggression in rodents can be distinguished, was suggested by the group of de Boer and Koolhaas (de Boer et al. 2003; Natarajan & Caramaschi 2010; de Boer & Koolhaas 2005). In an aggression study in rats, they discovered that, while in animals showing normal, functional levels of aggression a positive correlation between aggression and 5-hydroxyindoleacetic acid (5-HIAA) levels, the main 5-HT metabolite, was observed in the cerebrospinal fluid (CSF), in animals that showed aggression outside of the normal range, reduced levels of 5-HT were found. The observed abnormal aggressive behaviour was defined as a strong reduction in ritualised introductory social behaviours upon repeated winning experience. The hyper-aggressive animals were unresponsive to social cues such as submissive postures and did not follow ritualised patterns of threat and retreat. For a comprehensive review of animal violence see (Natarajan and Caramaschi, 2010). Similar to the observations in rodents, in humans reduced 5-HIAA levels in CSF were, in particular, found in individuals showing higher impulsivity and aggressive behaviour (e.g.(Brown, Goodwin and Ballenger, 1979; Comai *et al.*, 2016)). Also in primates, changes in the 5-HT system were related to alterations in aggressive behaviours related to pathology (Lesch and Merschdorf, 2000). These findings suggest that 5-HT is one important molecular determinant of abnormal aggression.

The serotonin system and its impact on emotion regulation

5-HT, one of the brain’s monoamines, is known as an essential regulator of manifold emotional and social behaviours (Baldwin and Rudge, 1995; Hariri and Holmes, 2006; Canli and Lesch, 2007). The 5-HT system within the brain possesses a most complex and elaborate network of projections that ascend from the raphe nuclei and project to various forebrain and brainstem structures (Steinbusch, 1981). The raphe is organised into nine nuclei, i.e. B1-B9 (Dahlström and Fuxe, 1964) that can be divided into nuclei projecting towards the spinal cord and periphery (B1-3) and nuclei that project to most of the forebrain structures (B5-B9). B5-B9 can be further subdivided into DR and median raphe (MR) and B9, projecting towards almost all forebrain regions, including hypothalamus, amygdala, septum, hippocampus, diverse cortical structures and the olfactory bulb as depicted in figure 2 (Hensler, 2006; Lesch and Waider, 2012).

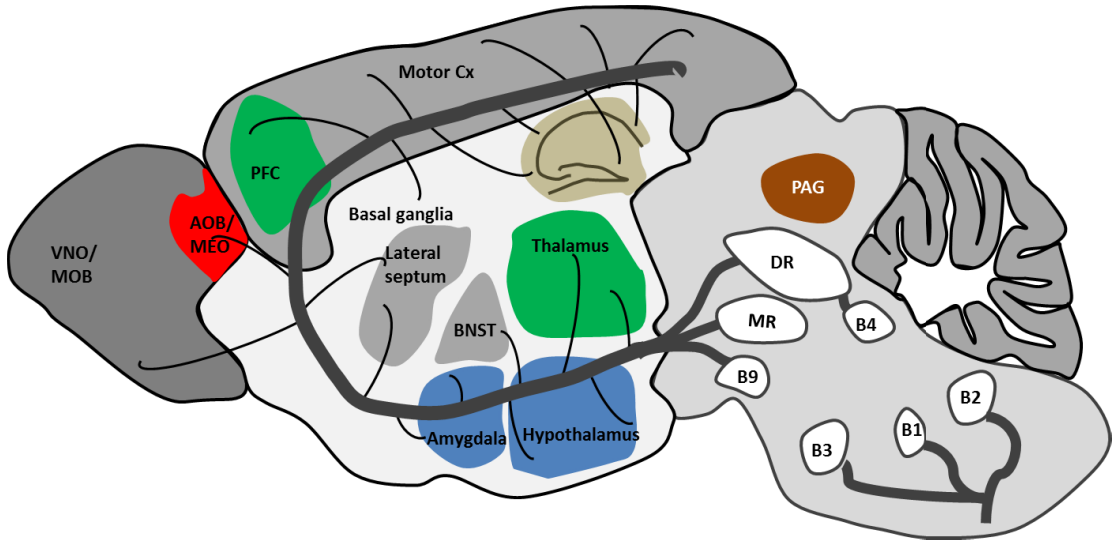

Figure 2 Projections of the serotonin system, ascending from diverse raphe nuclei. Serotonin (5-HT) is one of the brain's monoamines and possesses a most complex and elaborate network of projections. These projections are ascending from the raphe nuclei to various forebrain and brainstem structures. The raphe is organised in nine nuclei, B1-B9 that are projecting towards the spinal cord and periphery (B1-3) and most of the forebrain structures (B5-B9). The dorsal raphe (DR) comprises the nuclei B6 and B7 and median raphe (MR) the nuclei B5 and B8. Both are projecting towards numerous brain structures of the limbic system, diverse cortical structures and the olfactory bulb, containing the vomeronasal organ (VNO)/main olfactory bulb (MOB) as well as the accessory olfactory bulb (AOB) and the main olfactory epithelium (MOE) that project to the amygdala and via this route to the limbic system. B9 is a lateral extension of the MR. PAG=periaqueductal grey, BNST= bed nucleus of the stria terminalis, motor Cx= motor cortex.

With regard to aggressive behaviour, the 5-HT system represents one of the first identified heritable factors. In a Dutch family, where males showed a wide spectrum of abnormal violent behaviours ranging from arson to rape analysis of 24-hour urine samples suggested a markedly dysfunctional monoamine metabolism. Follow up examination revealed a deficiency in the function of monoamine-oxidase A (*MAO-A*), the enzyme degrading 5-HT (Brunner *et al.*, 1993). A point mutation in exon VIII of the *MAO-A* gene that was found in all affected male individuals, was identified as the cause of this metabolic deficiency. The *MAO-A* gene in humans is located on the X-Chromosome, rendering affected males with a full deficiency. Since the identification of the first family, further work identified additional families carrying similar mutations in the *MAO-A* gene and confirming the phenotypic consequences observed in the initial study (Piton *et al.*, 2014; Palmer *et al.*, 2016). Similarly, in non-human primates, polymorphisms in genes, related to 5-HT system functioning, such as the *MAO-A* gene and the 5-HT transporter (*5-HTT*) gene, have been associated with individual and species-related differences in aggression (Wendland *et al.*, 2006; Kalbitzer *et al.*, 2016). Furthermore, studies in mice, applying selective breeding paradigms to establish hyper-aggressive offspring (Lagerspetz, 1961; Oortmerssen and Bakker, 1981; Cairns, MacCombie and Hood, 1983), showed changes in various physiological parameters, related to the 5-HT system, concomitant with highly aggressive behavioural characteristics (Veenema *et al.*, 2003, 2005).

Overall, the 5-HT system comprises several levels of effect that are time- and brain region-specific and susceptible towards disturbances by genetic or environmental components (Gaspar, Cases and Maroteaux, 2003). The synthesis of 5-HT in the brain is restricted to raphe neurons, where a two-step reaction is driven by the rate limiting enzyme tryptophan hydroxylase 2 (TPH2) (Walther, Peter and Bashammakh, 2003; Gutknecht *et al.*, 2009). Upon its synthesis, 5-HT is actively transported into vesicles by vesicular monoamine transporter (VMAT) 1 and 2. There, it is stored until an action potential initiates its exocytotic release into the synaptic cleft. Following release, the physiological effects of 5-

HT are mediated by several 5-HT receptors that are located at the pre- and post-synapse. There are at least 14 structurally and pharmacologically distinct 5-HT receptors (5-HTRs) (Barnes and Sharp, 1999). Subsequent to receptor binding, presynaptic 5-HTT terminates the effects of 5-HT on any receptor by 5-HT reuptake into the presynaptic cell, where it is partially degraded by MAO-A, or reloaded into vesicles. By means of reuptake the 5-HTT is regulating the extracellular 5-HT concentration, thereby terminating transmission at 5-HT receptors and sustaining homeostasis (Torres et al. 2003). Next to associations with basic physiological functions, 5-HT modulates the activity of other neuronal networks, and shapes and regulates cognition and complex emotional behaviours in interaction with environmental stressors (Lesch and Merschdorf, 2000; Lesch and Waider, 2012).

Besides the selection breeding models of aggression, genetically engineered, rodent models were used to provide further insight on the role of 5-HT in the physiology of emotion regulation. Below, several models of deficits in major, regulatory components of the 5-HT system are discussed. In all of these models, homozygous knockouts (-/-) display substantial changes in terms of physiology, as well as emotional processing and associated behaviours, concomitant with profound changes in 5-HT signalling, regulation and abundance even under baseline conditions, when compared to wildtype (+/+) animals (Murphy and Lesch, 2008; Lesch and Waider, 2012; Mosienko *et al.*, 2014). The most prominent features of the described 5-HT-related mouse models are displayed in Figure 3. Effects in heterozygous (+/-) animals are usually more subtle and strongly dependent on the context, such as early-life stress exposure (e.g. (van den Hove *et al.*, 2011)).

One of the most prominent mouse models, showing aggression-related behavioural changes, comprises a **depletion of TPH2**. TPH2 is member of a family of monooxygenases (Fitzpatrick, 1999). It is the brain-specific form of the tryptophan hydroxylases and was detected and described in 2003 (Walther, Peter and Bashammakh, 2003). In the adult mammalian brain *Tph2* is expressed exclusively within the raphe nuclei (Gutknecht *et al.*, 2009). A depletion of this gene renders the organism completely derived of brain 5-HT. Studies investigating murine models of brain 5-HT deficiency, in mouse lines, depleted of TPH2, showed a variety of behavioural changes, such as hyperactivity and alterations in anxiety-related behaviours that were accompanied by a multitude of physiological phenotypes (reviewed in (Lesch *et al.*, 2012; Mosienko *et al.*, 2014)), as well as altered HPA axis activity (Gutknecht *et al.*, 2015). One particularly prominent phenotype was described as increased aggression. Various studies reported an increased number of attacks, prolonged attack duration, and shorter latency to fight in the resident intruder paradigm (Angoa-Pérez *et al.*, 2012; Mosienko *et al.*, 2012). Furthermore, *Tph2*$^{-/-}$ mice displayed compulsive behaviours, as determined in the nestlet shred and the marble burying test (Angoa-Pérez *et al.*, 2012). Administration of the immediate 5-HT precursor 5-hydroxytryptophan (5-HTP), circumventing TPH2 activity, was able to attenuate the observed aggression (Angoa-Pérez *et al.*, 2012). Similar, dramatic alterations in behaviour were observed in a model, **deficient for the *Mao-a* gene**. Animals, deprived of MAO-A, showed increased levels of 5-HT during early-life, but not during adulthood (Cases *et al.*, 1995; Holschneider *et al.*, 2001). Nevertheless, null mutants showed increased territorial aggression in the resident-intruder test and low levels of social investigation. In line with this, an increased number of bite wounds was observed in group-housed *Mao-a*$^{-/-}$ males (Cases *et al.*, 1995; Popova and Skrinskaya, 2001; Vishnivetskaya and Skrinskaya, 2007). Depression- and anxiety-like behaviours were reduced in MAO-A-deficient animals (Cases *et al.*, 1995), while they showed an enhanced emotional learning in fear conditioning and step-

down avoidance (Kim, Shih and Chen, 1997). Other groups found altered anxiety and exploration-related behaviours, suggesting that the lack in MAO-A is leading to impaired appraisal (Popova and Skrinskaya, 2001; Godar and Bortolato, 2011). Accordingly, they found that potentially higher threats, such as predator odour, induced a reduction of defensive burying. These findings were not related to sensory deficits (Godar and Bortolato, 2011). Only minor changes were observed concerning autonomic physiology of MAO-A-deficient animals, namely a decrease in blood pressure and heart rate in a restraint resting state (Holschneider *et al.*, 2000). Mice comprising a disruption of the ***5-Htt* gene** were found to have an altered 5-HT homeostasis and stress reactivity (reviewed in (Murphy and Lesch, 2008)). On the behavioural level, these mice display reduced locomotor activity, increased anxiety and decreased aggression. All in all, these data imply that decreased brain 5-HT levels (TPH2 deficiency) always lead to increased aggression, while it seems that altered 5-HT signalling (MAO-A or 5-HTT deficiency) can lead to increased or decreased aggression-related behaviours.

One possible explanation for this observation is the, previously mentioned, multi-layered nature of 5-HT effects. This multitude of effects might be mediated in a spatio-temporal manner through the complex interplay of diverse 5-HT receptors (Gaspar, Cases and Maroteaux, 2003; Wirth, Holst and Ponimaskin, 2017). Therefore, the effects of a dysregulated 5-HT system can exert a multitude of small individual effects on different brain circuitries, dependent on the affected component of the 5-HT system and timing throughout development (Gaspar, Cases and Maroteaux, 2003; Migliarini *et al.*, 2013). For example, when examining 5-HT receptor expression, *Tph2*$^{-/-}$ mice were shown to have an increase in 5-HTR1a and 1b abundance and binding capacity (Gutknecht *et al.*, 2012; Araragi *et al.*, 2013). *Mao-a*$^{-/-}$ mice have been associated with an increase of *5-Htr1a* mRNA expression in the FC and the amygdalar complex (Popova, Naumenko and Plyusnina, 2007). A radiolabelling study in these animals suggested a modest down-regulation of postsynaptic 5-HTR1a, 5-HTR2a and 5-HTR2c in a region-specific manner(Shih *et al.*, 1999). In *5-Htt*-deficient animals the density of the 5-HTR1a was significantly decreased in the DR and increased in the hippocampus in comparison with 5-HTR1a abundance in wildtype mice (Fabre, Beaufour and Evrard, 2000). Another observation in these animals was a marked 5-HTR1a desensitization in *5-Htt*$^{-/-}$ mice (Li *et al.*, 1999; Araragi *et al.*, 2013).

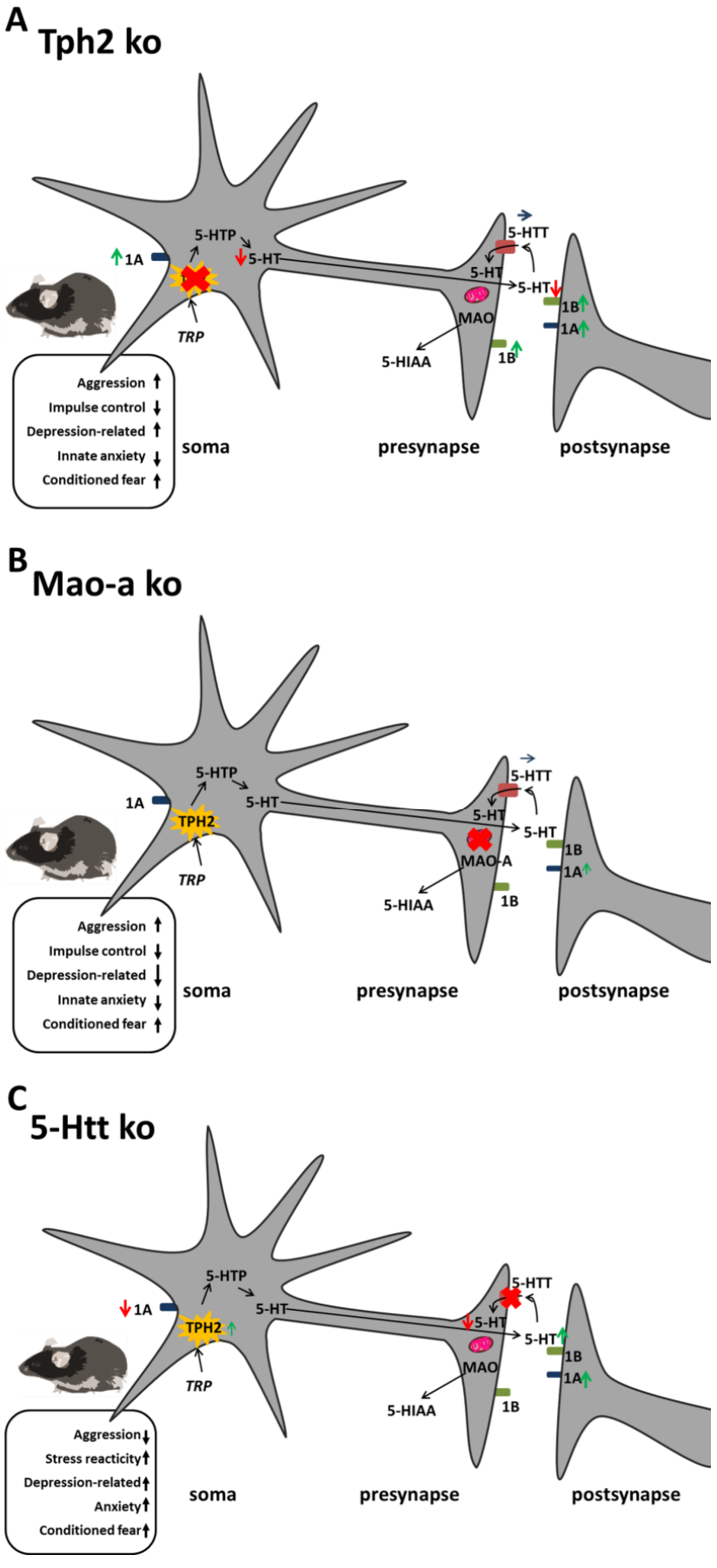

Figure 3 Overview of serotonin system modifications and their behavioural and molecular consequences. (A) Tryptophan hydroxylase 2 (*Tph2*) -deficient mice, completely deprived of brain serotonin (5-HT) show and increase in aggression and an upregulation of 5-HT1a and 1b receptors. (B) Monoamine oxidase A (*Mao-a*) -deficient mice show an increase in aggression and increased expression of the postsynaptic 5-HT1a receptor. (C) The 5-HT transporter (*5-Htt*) -deficient mouse line shows a decrease in aggression and brain region-dependent changes in 5-HT1a receptor abundance and reactivity. TRP = tryptophan, 5-HTP = 5-hydroxytryptophan, 5HIAA = 5- hydroxyindoleacetic acid.

The 5-HTR1a can act as both auto- and hetero-receptor and is closely related to the 5-HTR1b. Both receptors are expressed in 5-HT-, as well as in non-5-HT-neurons (Barnes and Sharp, 1999). The 5-HT1a autoreceptor is located in the soma and dendrites of 5-HT neurons, where it inhibits 5-HT neuron firing by inducing hyperpolarisation (Sotelo and Cholley, 1990; Barnes and Sharp, 1999). The 5-HT1a heteroreceptor mediates neuronal inhibition by coupling to the G protein-gated inward rectifying potassium channel subunit 2 (GIRK2) (Lüscher *et al.*, 1997). The 5-HT1b autoreceptor is located at axon terminals of 5-HT neurons and its activation results in an inhibition of transmitter release (Boschert *et al.*, 1994; Ghavami *et al.*, 1997). *5-Htr1a* and *1b* knock out mice show a variety of behavioural changes compared to control animals. However, while *5-Htr1a* knockout mice displayed an increase in anxiety-like behaviours and a decrease in aggression, *5-Htr1b* knockout mice were less anxious and more aggressive (Zhuang *et al.*, 1999; Gross *et al.*, 2000; Klemenhagen *et al.*, 2006). Interestingly, the hyper-anxious phenotype that is observed in *5-Htr1a* knockout mice is suggested to be established during development, as conditional 5-HTR1a depletion in adulthood has no such effect (Gross *et al.*, 2002; Lo Iacono and Gross, 2008). This suggestion of early establishment of behavioural deviation is further indicated by observations in the previously discussed models of 5-HT system manipulation. For example, in MAO-A-deficient animals, the normalisation of 5-HT levels during development was sufficient to restore normal brain development (Cases *et al.*, 1996). Moreover, in adult MAO-A-deficient animals a normal 5-HT level is observable, which might be accountable to compensatory activity of MAO-B (Cases *et al.*, 1995).

The idea of a critical period was further supported by general observations regarding the 5-HT system and its development. 5-HT can be detected early during foetal development (Golden, 1973) and modulates basic, morphogenetic processes including cell proliferation, migration and circuitry wiring (Gaspar, Cases and Maroteaux, 2003). And, while during prenatal development and basic wiring the availability of 5-HT from external sources such as maternal and placental 5-HT is able to complement the embryonic 5-HT system (Côté and Fligny, 2007; Bonnin *et al.*, 2011), the early postnatal period is critically vulnerable to endogenous 5-HT deficiency, which is resulting in altered innervation of forebrain regions, such as the suprachiasmatic and thalamic paraventricular nuclei, the nucleus accumbens and the hippocampus (Migliarini *et al.*, 2013). Taken together, these findings suggest that the fundamental wiring of the brain occurs prenatally, is including processes related to development of the 5-HT system itself and supported by the maternal/placental 5-HT system, while the fine-tuning of the circuitry ensues postnatally and is highly dependent on endogenous 5-HT signalling. Therefore, any impairment of 5-HT homeostasis throughout development becomes a potential source of pathological consequences, even in the absence of changes in 5-HT levels in adulthood.

Developmental stress as a modulatory factor of susceptibility

The developmental period is particularly prone to environmental influences and can alter subsequent susceptibility to disease (Lupien *et al.*, 2009). Studies, investigating the effects of adverse events during pregnancy and early life on human development, showed that chronic exposure to stress throughout the prenatal period, infancy and childhood affects mental health and underlying brain molecular factors (van den Bergh *et al.*, 2017). Such data was collected mostly by self-report, in relation to a clinical diagnosis of the pregnant mothers or in the context of external events, such as natural

catastrophes or loss of husband or kin, during pregnancy. For example, offspring of mothers with prenatal, maternal depression displayed aberrant behaviours at the age of 12 months, with regard to aggression-, anxiety- and activity-related behaviours (Gerardin *et al.*, 2011). Some of these traits were more pronouncedly changed in male, others in female offspring. Similarly, a report by mothers and teachers on child emotional and behavioural problems at the age of 10-11 years, suggests a modulatory role of antenatal maternal distress and the offspring's emotions and behaviour (Leis *et al.*, 2014). It has to be mentioned that these effects were lost, with regard to the teacher-based evaluation, when the investigated model was corrected for further psychological variables of the mothers. Furthermore, effects of early adversity can be observed throughout adolescence and adulthood. Maternal pre- and post-natal anxiety exposure increased the risk for onset of conduct disorder in male, and decreased the risk for the onset of conduct disorder in female offspring at the age of 16 (Glasheen *et al.*, 2013). In the same study, high depression levels of the mothers during pregnancy were related to lower anxiety symptoms in male infants, while, in another study, increased maternal cortisol levels during pregnancy were related to affective problems in female infants (Buss *et al.*, 2012). Another study, in 21 year old offspring, associated internalizing and externalizing problems with high levels of maternal, subjective depressive symptoms, anxiety and stress during pregnancy (Betts *et al.*, 2015). In a Danish registry study on bereavement, a relation between prenatal bereavement and the risk to develop attention deficit hyperactivity disorder (ADHD) was found (Li *et al.*, 2010). A Swedish registry study was able to replicate these effects showing an association between, amongst others, ADHD symptoms in adult offspring and third trimester stress (Class *et al.*, 2014). In this study, also postnatal bereavement was investigated and showed effects on the incidence of suicide and the risk for developing autism spectrum disorder. Besides bereavement, further potential unfavourable factors during postnatal development are abuse, neglect and poor parental care. For example, children brought up by depressive mothers showed a lack in empathy at the preschool age (Jones, Field and Davalos, 2000) as well as altered cortisol measures, predictive of self-reported depression during adolescence (Halligan *et al.*, 2007). Children that had been abused or neglected during early life showed an increased risk for adult deviant behaviours, including suicide attempts, antisocial personality disorder and alcohol abuse (Widom, 1989, 2000). Studying early environment revealed also beneficial effects of positive early environment, modulating the outcome of prenatal stress (PS) exposure in young infants (Pickles *et al.*, 2017). Interestingly, the effects of maternal cortisol, measured during pregnancy, on offspring behaviour and their relation to the type of stress, experienced by the mother, are inconclusive (reviewed in (van den Bergh *et al.*, 2017)). Taken together, observations in humans are suggesting that the specific effects of early adversity on brain development and through such on behaviour and cognition are not only dependent on the stress exposure per se, but are affected by manifold factors, such as timing and duration of the exposure, as well as the nature of the experienced stress.

In macaques, similar to the human studies, early adversity was shown to influence diverse behaviours such as exploration and social behaviours in the affected animals as well as HPA axis activity (Schneider *et al.*, 2002; Dettmer *et al.*, 2016). As in the human studies, the effects of early adversity were strictly dependent on context and nature of the experienced environment.

Pioneering work in the field of early adversity was done in rodents. In line with later findings in humans and non-human primates, studies in rats showed that early-life experiences have the capacity to affect

stress response and behaviour later in life (Thompson, 1957; Levine, 1967). GCs have been suggested, as one major effector of adverse prenatal conditions (Moisiadis and Matthews, 2014). During early gestation the transfer from maternal GCs to the foetus is kept low by the activity of the 11β-hydroxysteroid dehydrogenase 2 (Yang, 1997). The activity of this enzyme is decreased during late gestation, allowing increased transfer of active GCs from mother to foetus (Thompson, Han and Yang, 2002). In the foetal brain, GCs affect a number of developmental processes, including neurogenesis, gliogenesis, and synaptogenesis as well as the epigenome and can induce alterations in sensitivity and activity of the HPA axis (Moisiadis and Matthews, 2014). Next to the foetal exposure to GC, maternal behaviour during the early postnatal phase was found to be relevant for stress susceptibility later in life (Levine, 1967). Effects of maternal care were associated with altered 5-HT functioning and HPA axis programming (Hellstrom *et al.*, 2012). Next to the type of stress, the observed behaviours are also dependent on the time-point of stress exposure. Furthermore, the investigated species as well as the genetic make-up determine the outcome, supporting the previously mentioned complex nature of early-life programming (Lupien *et al.*, 2009). Detailed phenotyping in the animals, exposed to early adversity, allowed for an association of early environment with a multitude of behavioural outcomes (Fumagalli *et al.*, 2007).The observed behavioural consequences of such early adversity were manifold and mostly accountable to altered stress reactivity. Next to anxiety and depressive-like behaviours, social behaviour was most prominently affected by either type of early adversity. Following PS, Sprague-Dawley rats were found to display altered social behaviours, i.e. male rats showed decreased bodily sniffing while females under the same conditions showed an increase in social exploration (Schulz *et al.*, 2011). Similarly, adult, male Sprague-Dawely rats, previously exposed to PS, showed reduced social interaction and also decreased quality of the social interaction behaviour (P. R. Lee *et al.*, 2007). Moreover, Lee and colleagues showed that *Oxt* mRNA was reduced in the PVN of PS animals, while increased OXTR binding was observed in the central amygdala. Administration of OXT into the central amygdala reversed the social incompetence, observed in PS animals. In another study, male Wistar rats that were subjected to PS displayed impaired social memory and interaction (de Souza *et al.*, 2013). Increased aggressiveness was observed only, when stressed pups were brought up by stressed dams. When cross-fostered by a non-affected mother this effect was abolished. Moreover, PS and cross-fostering had a complex effect on the abundance of OXT- and AVP-positive cells in the paraventricular nucleus (PVN) of the hypothalamus (de Souza *et al.*, 2013). In C57BL/6 mice, PS increased lunges and attacks towards an intruder when compared to control animals, while DBA/2J male mice did not show any changes in aggression behaviour following PS, when compared to controls (Kinsley and Svare, 1987). Following maternal separation (MS), male Wistar rats showed increased aggression in juvenile play-fighting (Veenema and Neumann, 2009) and in adult inter-male encounters (Veenema *et al.*, 2006) as well as increased AVP levels in the PVN. In C57BL/6J males, MS suppressed aggressive behaviours at a juvenile age, but had no effect on social investigation (Tsuda, Yamaguchi and Ogawa, 2011). Concomitant with decreased aggression, MS offspring had lower plasma testosterone levels and altered OXT and AVP immunoreactivity in the PVN. These results were confirmed in adult males of the same strain (Veenema, Bredewold and Neumann, 2007). In this study, they furthermore related MS to increased AVP immunoreactivity in the PVN.

All in all, studies in various species on the effects of early adversity, suggest that, dependent on the timing of stress exposure, type of stress, further life experiences and genetic background, programming effects will vary, resulting in a wide spectrum of molecular and behavioural consequences. One of the most pronounced behavioural consequences of such programming is altered aggression-related and social behaviour, concomitant with AVP and OXT expression changes. Thus, even though the modulatory role of early adversity is indisputable, a notable heterogeneity in phenotypic consequences becomes apparent.

Gene-by-environment interactions – stress and the 5-HT system

Molecular and functional correlates of early adversity have been shown to be as multifaceted as the observed behavioural alterations. Studies in various species suggest an altered functioning of the HPA axis as the most prominent consequence of early adversity, which is also related to the observed behavioural alterations (reviewed e.g. in (Sanchez *et al.*, 2001)). Moreover, the investigated aetiology of early adversity, as well as its pathological outcome in interaction with altered 5-HT system functioning, suggests a modulatory role of 5-HT signalling on HPA axis function and stress reactivity, in this respect (Lesch and Merschdorf, 2000). In humans, the association between early-life maltreatment in boys and conduct disorder, antisocial personality, and adult violent crime were shown to be contingent upon low expression of the *MAO-A* gene (Caspi *et al.*, 2002). Further findings highlighted the important role of the short variant of the *5-HTT* gene-linked polymorphic region (*5-HTTLPR*), which has been associated with reduced expression of *5-HTT in vitro* (Heils *et al.*, 1996). Stressful life events increased the incidence of anxiety and depression-related traits in carriers of the short allele, while homozygous long allele carriers showed no such propensity (Caspi *et al.*, 2003). In macaques, an analogous variant of the 5-HTTLPR was associated with altered, central 5-HT functioning, dependent on early adversity (Bennett *et al.*, 2002). A mouse study by our group that investigated the interaction of *5-Htt* genotype and PS, identified a 5-HTT-dependent effect of PS on depressive-like behaviour, in particular in female offspring (van den Hove *et al.*, 2011). Furthermore, differential susceptibility to the effects of PS was observed. This differential susceptibility was independent of the *5-Htt* genotype, while associated molecular changes were found to be *5-Htt* variant-specific (Jakob *et al.*, 2014). In general, mice showing a reduced *5-Htt* expression have been found to display altered stress reactivity, with reduced basal plasma corticosterone levels and an increased acute response to stress (Murphy and Lesch, 2008). Mice, lacking 5-HT, showed altered neuroendocrine signalling and related behaviours in a sex-specific manner. Both, male and female *Tph2*$^{-/-}$ mice, showed altered faecal corticosterone metabolite (FCM) and plasma corticosterone levels when compared to their wildtype counterparts. Following stress-induced hyperthermia and behavioural testing, male *Tph2*$^{-/-}$ mice remained unaffected in comparison to wildtype and *Tph2*$^{+/-}$ males, while female *Tph2*$^{-/-}$ mice showed a lower baseline FCM and increased levels during the initial phase of chronic mild stress when compared to wildtype and *Tph2*$^{+/-}$ females (Gutknecht *et al.*, 2015). Throughout early life, *Tph2* knock-in mice, with lower 5-HT levels in the brain, had been shown to remain unaffected by stress exposure, albeit their behaviour, observed under baseline conditions, was indicative of effects, observed in WT stressed offspring (Wong *et al.*, 2015).

Further work towards understanding the mechanisms, underlying the observed changes in early adversity, suggested that it exerts its effect by promoting or inhibiting the connectivity amongst limbic brain structures, involved in the regulation of HPA axis activity and associated behaviours. In human studies, early adversity altered functional connectivity between relevant, regulatory regions and their volume. For example, maternal, prenatal, but not postnatal depression was associated with a higher connectivity between the left amygdala and limbic structures such as the ventromedial prefrontal cortex (vmPFC), in 6 months old infants (Qiu *et al.*, 2015). Increased maternal cortisol levels, in early pregnancy, were associated with a larger volume of the right amygdala and affective problems in girls (Buss *et al.*, 2012). In a cohort of healthy, young women, prenatal stress was not associated with trait anxiety, depressive symptoms or state anxiety, but a decreased grey-matter volume in both amygdala nuclei (Favaro *et al.*, 2015). Moreover, grey matter volume correlated also with anxiety traits and depressive symptoms. In this study, prenatal stress, as well as depressive symptoms were, furthermore, related to altered connectivity between some of the observed regions. Similarly, postnatal adversity resulted in distinct structural and functional changes. For example, smaller hippocampal and PFC volumes, altered amygdalar volume as well as decreased activation of the hippocampus and PFC, and increased activation of the amygdala were observed as a consequence of adverse, early-life events (reviewed in (Krugers *et al.*, 2017)). In addition, early adversity was found to alter resting-state functional brain connectivity of limbic structures such as the amygdala and hippocampus with PFC regions (Herringa *et al.*, 2013). This was partially sex-specific and associated with adolescent internalizing problems. One potential candidate in mediating these manifold effects of early adversity on brain connectivity and function is the 5-HT system. As previously discussed, genetic variation of the 5-HT system has been identified as an important modulator of experienced stress in human and non-human primates as well as in rodents. Furthermore, 5-HT signalling was identified as crucial developmental agent. In human cohorts, polymorphisms regarding the 5-HT system were associated with altered amygdalar reactivity to negative stimuli (Canli *et al.*, 2005; Canli and Lesch, 2007). Similarly, in mouse studies, genetic variation of the 5-HT system were associated with altered brain development and, consequently, morphology (Gaspar, Cases and Maroteaux, 2003).

In addition to its mediatory effects, 5-HT function has been shown to be directly affected by early adversity. For example, in 12 weeks-old macaques, specific stressful events during early infancy were associated with decreased *5-HTT* expression in peripheral blood upon separation from the mother (Kinnally *et al.*, 2010). This observation was unrelated to the *5-HTTLPR* genotype, suggesting a direct effect of early adversity on *5-HTT* expression. Moreover, in adolescent monkeys, life-long maternal deprivation resulted in a decreased binding capacity of the 5-HTT in relevant brain regions, such as the raphe, hippocampus and amygdala (Ichise, 2006). In mice, exposed to PS, increased immunoreactivity of 5-HT and TPH was detected in the DR (Miyagawa *et al.*, 2011). Moreover, a recent study showed an association of PS with the up-regulation of *5-Htr2a* mRNA expression in the FC of male adult CD1 mice (Holloway *et al.*, 2013). In 4 weeks old, male and female, 344 Fischer rats, reduction of 5-HTR1a within the ventral hippocampus was observed (Van den Hove *et al.*, 2006). Prenatal dexamethasone treatment, which mimics PS, was shown to induce increased anxiety and despair-related behaviour in female, but not male, offspring, exposed to dexamethasone. (Hiroi *et al.*, 2016)Furthermore, they observed that dexamethasone during gestation induced increased *Tph2* mRNA expression, during early postnatal life, and decreased *Tph2* mRNA expression in adulthood, in

the caudal DR of female rats. Our group found changes in 5-HT immunoreactivity in diverse brain regions of male rats that were exposed to PS, with a decrease in 5-HT, and 5-HT-positive cells in the DR and an increased 5-HT immunoreactivity in forebrain regions such as the PFC in male offspring (van den Hove *et al.*, 2014). In the same study, TPH2 immunoreactivity was found to be increased in the hippocampus of PS animals, both male and female. Further studies report effects of MS on the expression of 5-HT system components such as 5-HTT, TPH2 and MAO-A. Wong and colleagues found decreased 5-HT levels in FC, striatum and brain stem tissue and an increased MAO-A protein level and *Mao-a* mRNA expression during adolescence and adulthood in the striatum and brain stem of mice exposed to MS (Wong *et al.*, 2015). Furthermore, MS decreased 5-HT abundance in the hippocampus and PFC , compared to controls, in a sex-specific manner (Matthews *et al.*, 2001; J.-H. Lee *et al.*, 2007). In male rats, MS-induced reduced 5-HT immunoreactivity in the hypothalamus, which correlated negatively with the display of inter-male aggressive behaviour (Veenema *et al.*, 2006). Altogether, these findings suggest profound alterations in 5-HT functioning in response to early-life stress. Next to direct effects, early adversity was found to affect 5-HT functioning in a context-specific manner in adult rats suggesting a programming effect. Changes in *5-Htt* and *Tph2* expression in specific subregions of the DR were observed following social defeat during adulthood, only if animals had experienced MS during early life (K. L. Gardner *et al.*, 2009; Katherine L. Gardner *et al.*, 2009). Even transgenerational effects of MS on the 5-HT system were observed: a decrease in 5-HTR1a binding was found in several relevant brain regions, including DR of male F2 offspring of C57BL/6 males, subjected to unpredictable MS combined with unpredictable maternal stress (Franklin *et al.*, 2011). Accordingly, they observed increased 5-HT levels in projection areas of the DR, i.e. the FC.

As previously suggested, the HPA axis and GCs are key players of early-life-related programming. GCs function as mediators and integrate environmental challenges to generate physiological and behavioural responses (McEwen, 2003). In this context, they were shown to interact directly with 5-HT functionality. For example, an effect of altered GC signalling on the expression, abundance and functionality of 5-HT receptors was observed in transgenic mice and rats with manipulated adrenal functions (Chalmers *et al.*, 1993; Holmes *et al.*, 1995; Meijer *et al.*, 1997; Meijer, Van Oosten and De Kloet, 1997). Dexamethasone treatment during the early postnatal period increased *Mao-a* expression and reduced 5-HT levels in several relevant brain regions (Wong *et al.*, 2015). This was concomitant with reduced GR levels. Subsequent *in vitro* experiments, where exposure of SH-SY5Y cells to dexamethasone was sufficient to increase MAO-A expression, support the idea of a glucocorticoid-dependent regulation of MAO-A expression. Chronic corticosterone administration via the drinking water induced an upregulation of TPH2 protein levels in the DR in the light phase, abolishing the diurnal expression pattern of this enzyme (Donner, Montoya, *et al.*, 2012). Thus, taken together, observations in several species are supporting the notion of an interactive mechanism of genetic predisposition, in particular of components of the 5-HT system, and effects induced by the exposure to adversity on emotion-related behaviours (Booij *et al.*, 2015). One mechanism of such interactions and its potential link to 5-HT-related pathological aggression will be discussed in the next section.

The eclectic roots of violence and their distinctive nature

Several elegant experiments by Donner and colleagues related early-life priming to later life stress susceptibility and showed that this interaction was mediated via a 5-HT-dependent pathway. In a first experiment they showed that priming the amygdala with corticotrophin releasing hormone (CRH) receptor (CRHR) agonist, resulted in increased expression of *Tph2* mRNA in subregions of the DR, including the ventrolateral PAG (vlPAG) (Donner, Johnson, *et al.*, 2012). Upon corticosterone exposure via the drinking water an increase in TPH2 during the light phase was observed in the dorsal and ventral DR (Donner *et al.*, 2016). In this context, a stress-dependent increase in TPH2 activity in the dorsal and caudal DR following acoustic startle was observed. In the same study, Donner and colleagues showed that pharmacological CRHR2 blockade prevented this increase of TPH2 activity, whereas CRHR1 blockade enhanced TPH2 activity. Interestingly, this stress dependent activity in dorsal and caudal DR was associated with TPH2 activity in various target areas of 5-HT projections. In light of this association, Donner and colleagues suggested context-specific activation of projections towards amygdalar subnuclei. Both basolateral and central amygdala are innervated by DR neurons (Commons, Connolley and Valentino, 2003; Hale *et al.*, 2008). Next to CRHR, also GRs are expressed in the DR (Aronsson *et al.*, 1988), where they have been shown to interact with 5-HT functioning (Héry *et al.*, 2000). Deletion of DR GRs was shown to impair HPA axis feedback inhibition and to promote active coping (Vincent and Jacobson, 2014). All in all, these data underscore the modulatory role of 5-HT in the mediation of early adversity and indicate reciprocal 5-HT/HPA interactions involving e.g. specific subsets of 5-HT DR neurons and CRH expressing neurons of the central amygdala (Hale *et al.*, 2008). Interestingly, the same players implicated in this interaction seem to be crucially involved in the regulation of the panic response and stress-induced hyperactivity (reviewed in (Paul *et al.*, 2014)). Additionally, a recent publication was able to identify a specific subset of raphe serotonergic neurons, to be critically involved in the modulation of aggression (Niederkofler *et al.*, 2016). In their study, Niederkofler and colleagues showed that the activity of Pet1 positive 5-HT neurons, expressing either dopamine receptor D2 (DRD2) or dopamine receptor D1a (DRD1a) is crucial in regulating aggression in mice. Interestingly, DRD2 5-HT cell silencing also evoked novelty-induced hyperarousal. Retrograde tracing, in this study, revealed that targets of these neurons comprised amongst others hippocampus, ventral tegmental area and olfactory bulb.

Thus, early-life adversity altering stress-reactivity in interaction with 5-HT might be mediating the observed deviation in aggression-related behaviours skewing the social reactivity towards a more offensive approach by promoting activity-related behavioural programmes. This is in accordance with studies linking hyper-arousal to exaggerated aggression. For example, in a study of social deprivation, exaggerated aggression and an increased GC release in response towards social challenge were observed (Toth *et al.*, 2011). Further findings were an increase of autonomic reactivity in the first aggressive encounter and a significant increase of defensive postures, while dominant postures were decreased when compared to control rats. Interestingly, such hyper-arousal-driven aggressive behaviour might be reinforced by a positive feedback of corticosterone release, facilitating the initiation of an aggressive encounter, which in turn facilitates corticosterone release and, as such, lowers the threshold of action (reviewed in (Kruk, 2014)). In humans, the clinical profile of sociopathy is associated with early adversity and deregulated amygdalar activation (Yildirim and Derksen, 2013). This altered

reactivity of the amygdala is suggested to be a consequence of a down-regulation of the cingulate-amygdala feedback circuit, which has been shown to be influenced e.g. by the low expressing variant of the *MAO-A* VNTR and the short allele of the *5-HTTLPR* (Pezawas *et al.*, 2005; Meyer-Lindenberg *et al.*, 2006) as well as, as discussed above, by early adversity. Thereby, HPA axis reactivity can be shifted in either direction, brought about by vmPFC signalling deficits, leading to mismatched appraisal and inappropriate behavioural responses (Yildirim and Derksen, 2013).

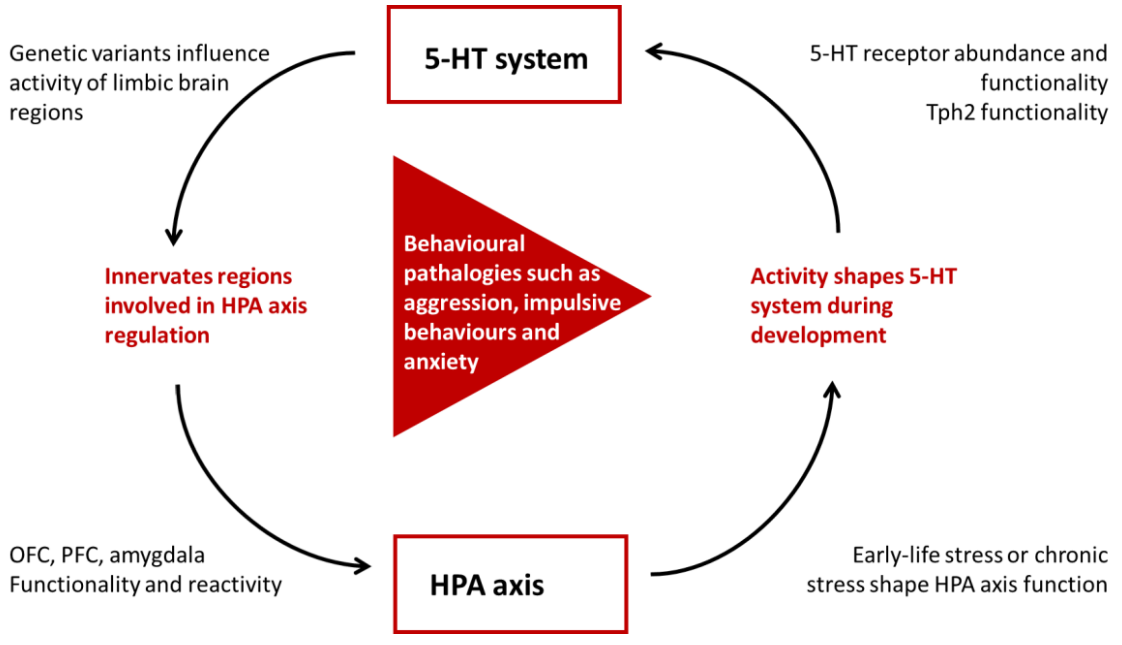

Figure 4 The developmental vicious cycle of violence. Genetic predisposition and early-life adversity reinforce each other's detrimental effects by creating adverse regulatory interactions and result in behavioural pathology related to exaggerated emotionality and aggression. 5-HT=serotonin, Tph2=tryptophan hydroxylase 2, HPA=hypothalamo-pituitary-adrena, OFC=orbitofrontal cortex, PFC=prefrontal cortex

Furthermore, it seems that the more callous form of aggression, often referred to as psychopathy, is not causally related to early stress. In contrast to sociopathy, psychopathy is related to hypo-arousal, which was suggested to be mediated by attenuated amygdalar reactivity, as seen in psychopaths (Yildirim and Derksen, 2013). Comparing adrenalectomized (ADX) rats with GC replacement (ADXr) to sham-operated rats and ADXr rats that receive an acute dose of corticosterone prior to testing showed a difference in qualitative measures of aggression for the ADXr, but not the ADXr animals that received acute corticosterone treatment. Both ADX groups showed a significant increase in attack counts (Haller, Van De Schraaf and Kruk, 2001). Moreover, animals treated in that way showed diminished autonomic arousal and social deficits (Haller and Halasz, 2004) and while normal aggression correlated negatively with activation of DR serotonergic neurons, no correlation could be observed in the ADXr animals displaying abnormal attack patterns (Haller, Tóth and Halász, 2005). Furthermore, an effect of corticosterone on the inhibitory effect of serotonergic signalling was observed (Stutzmann, McEwen and LeDoux, 1998). This, in turn, was impacting on excitatory transmission in auditory afferents to the lateral amygdala. In this study they showed that ADX rats without replacement, adrenalectomy abolished the inhibitory effect of 5-HT completely as did ADXr with low levels of corticosterone replacement. Only high levels of corticosterone were able to reinstate the inhibitory effect on amygdalar activity. Taken together, these findings suggest an attenuation of 5-HT-mediated modulation in animals with low and constant corticosterone. c-Fos imaging in ADXr rats with low GCs,

associated with predatory-like, hypo-arousal-driven aggression in particular, revealed a pathway overlapping with the neural control of predatory actions, including the central amygdala, lateral hypothalamus and ventral PAG was activated (Tulogdi *et al.*, 2010, 2015). A similar experiment in socially isolated rats, a paradigm shown to introduce hyperarousal-driven aggression (Toth *et al.*, 2008), identified an increased activation of the medial and lateral orbitofrontal cortices, anterior cingulate cortex, bed nucleus of the stria terminalis (BNST), medial and basolateral amygdala, hypothalamic attack area, hypothalamic PVN and locus coeruleus (Toth *et al.*, 2012), supporting the distinctive nature of the described categories of aggression.

Overall, these observations suggest that early adversity is specifically related to a hyperactivity-driven form of exaggerated aggression and that this association might be mediated via the regulation of 5-HT signalling and subsequent down-stream events. The suggestion of a vital role of 5-HT signalling in mediating early-life influences is supported by various studies, investigating an interaction of early adversity and genetic variation in the 5-HT system (Figure 4).

Epigenetics, the missing link

As highlighted above, several examples of direct effects of either 5-HT on stress reactivity, or vice versa, suggest the involvement of a 5-HT/HPA reciprocal interaction in the mediation of early adversity-evoked, deviant aggression. But, while we were able to associate, for example, early adversity to changes in 5-HT system component expression, the underlying molecular changes that were able to bring about this alteration in expression remained elusive in most cases. Therefore, it seems important to devote future work in aggression research towards a more molecular approach, taking into account not only neuroendocrine and genetic factors, but also the regulatory interface, i.e. epigenetics. As previously mentioned, the epigenome is susceptible to environmental cues throughout life (Moisiadis and Matthews, 2014). Over the last decade, a considerable number of studies have focussed on elucidating the molecular mechanisms that allow for a dynamic regulation of the relatively stable genome. Epigenetic mechanisms were shown to play a crucial role in X-chromosomal inactivation (Mohandas, Sparkes and Shapiro, 1981), in maternal and paternal imprinting (Li, Beard and Jaenisch, 1993), as well as in cell differentiation and development (Razin and Riggs, 1980). All of these findings represent programming effects that occur early in development and were thus assumed to remain stable throughout life. The true potential of epigenetic mechanisms has only recently been uncovered, as delicately discussed in a review by Szyf (2014), where he outlines the importance of epigenetic mechanisms in dynamic brain processes and points out how changes in epigenetic programming can render individuals more or less susceptible to psychopathology.

Epigenetic modifications comprise a variety of modifications at nucleosome-forming proteins called histones as well as methylation and hydroxymethylation at the DNA strand itself. Nucleosomes represent the basic unit of DNA packaging with 150 bp of DNA wrapped around an octamer of histones. Each nucleosome consists of one pair of histone proteins H2A, H2B, H3 and H4. Each of these histone proteins possesses an N-terminal amino-acid “tail” that can be subjected to various epigenetic modifications such as acetylation or methylation. As a consequence of histone modifications, the configuration of chromatin is changed, rendering genes more or less accessible towards transcription factors and/or the transcription machinery (Kouzarides, 2007). Histone modifications are manifold and

so are their effects on the chromatin state. Amongst the most extensively studied histone modifications are the H3K4me1, H3K4me2 and H3K4me3 that are highly abundant at transcription start sites of known genes and correlate positively with gene expression (Barski *et al.*, 2007). Furthermore, H3K9me1, H3K27me1 and H3K36me3 have been shown to be implicated more with gene activation than silencing, whereas H3K27me2 and me3 correlate with gene silencing. H3K9ac is strongly associated with gene expression (Barski *et al.*, 2007). Histone modifications are highly flexible and induced by histone modifiers, such as histone acetyltransferases, histone deacetylases (HDACs) or histone methyltransferases (Struhl, 1998; Narlikar, Fan and Kingston, 2002). These enzymes actively facilitate or hinder access to the DNA and, thus, transcription (Struhl, 1998; Narlikar, Fan and Kingston, 2002). Moreover, histone modifiers themselves can be recruited by certain histone modifications. For example, H3K9 methylation recruits chromodomain-containing proteins, such as the heterochromatin protein1 (Bannister *et al.*, 2001; Lachner *et al.*, 2001), which is associated with the histone methyltransferase Suv39h1 (Lachner *et al.*, 2001), rendering the chromatin in a highly condensed state. To interrupt this state the binding of such complexes can be hindered by employment of other histone modifications, which in the case of HP1-binding is an additional phosphorylation at the H3S10 (Fischle *et al.*, 2005).

As mentioned previously, DNA methylation represents another form of epigenetic regulation. Methylation of the cytosine ring at position 5 (5-methylcytosine: 5mC), in mammals usually in the constellation of a cytosine-phosphate-guanine (CpG) site, influences the binding of chromatin modifiers, repressors, and transcription factors and as a result regulates gene expression (Watt and Molloy, 1988; Boyes and Bird, 1991; Hendrich and Bird, 1998; Ng *et al.*, 1999; Klose and Bird, 2006). Another mechanism of DNA methylation-mediated regulation is via CpG islands (Bird, 1986). A high C and G content, a high CpG frequency, and significant hypomethylation, define CpG islands and they mostly can be found in gene promoter regions as well as in the closer vicinity of gene promoters (Illingworth and Bird, 2009). DNA methylation is carried out by specific enzymes called DNA methyltransferases (DNMTs) that transfer the methyl group from the methyl donor S-adenosyl methionine to cytosine. There are three DNMT families, DNMT1, 2 and 3. Of these, DNMT1 is mainly responsible for the maintenance of DNA methylation (Gruenbaum, Cedar and Razin, 1982; Hermann, Gowher and Jeltsch, 2004). It prefers hemi-methylated DNA over unmethylated DNA, a finding that is supporting the hypothesis of its maintaining function (Bacolla *et al.*, 1999; Hermann, Gowher and Jeltsch, 2004). DNMT1 has been shown to interact with histone modifiers such as the histone methyltransferase Suv39h1 and HDACs (Robertson *et al.*, 2000; Rountree, Bachman and Baylin, 2000; Fuks *et al.*, 2003). Moreover, interactions with methylCpG binding protein 2 and different methylCpG binding domain protein (MBD) variants have been reported (Tatematsu, Yamazaki and Ishikawa, 2000; Kimura and Shiota, 2003). The DNMT3 family comprises 2 variants 3a and 3b that both function as *de novo* methyltransferases (Hermann, Gowher and Jeltsch, 2004). Just like DNMT1, DNMT3a and 3b were found to interact with histone modifying factors as well as factors influencing DNA methylation (Fuks *et al.*, 2001, 2003). While both the DNMT1 and 3 families' interactions and functions have been vastly described, the biological meaning of the DNMT2 family has not yet been determined (Hermann, Gowher and Jeltsch, 2004).

While histone modifications have been known to be dynamic from the start, DNA methylation had long been assumed stable, as a basis of cell fate and differentiation (Razin and Riggs, 1980; Stein *et al.*,

1982). However, recent findings provide evidence for postnatal *de novo* methylation and active DNA demethylation (Szyf, 2014). Moreover, DNMT3a and 3b were shown to be expressed in the adult hippocampus, where their expression correlated with the global 5mC level (Brown *et al.*, 2008). Furthermore, several potential mechanisms of active demethylation, such as the removal of the methylated cytosine by repair mechanisms have been suggested (Razin *et al.*, 1986; Jost, 1993; Guo, Su, *et al.*, 2011). One of the suggested mechanisms is based on the oxidation of the 5mC by the hydroxylase ten-eleven translocation methylcytosine dioxygenase 1 to 5-hydroxymethylcytosine (5hmC) followed by the base excision repair pathway (Guo, Su, *et al.*, 2011). In addition, direct removal of the hydroxymethyl group from the cytosine ring by DNMT 3a and 3b was shown in an *in vitro* experiment (Chen, Wang and Shen, 2012). Further research in the field also revealed that histone acetylation is driving DNA demethylation (Cervoni and Szyf, 2001). Overall these observations suggest that DNA methylation is not exclusively acting as a stable steady-state regulator like it does in differentiation, but can also be dynamically regulated upon environmental stimulation, as found by Guo and colleagues (Guo, Ma, *et al.*, 2011). The discovery of the dynamic features of DNA methylation introduced DNA methylation and demethylation as possible mediators of environmentally driven regulation of gene expression (Szyf, 2014). Life events of good or bad nature both have been shown to induce persistent changes in DNA methylation on single-gene level, as well as globally. The type and extend of these changes is not only highly dependent on individual genetic differences, but also on the developmental stage at which they occur. Comparing the outcome of early-life events and stress exposure at a later time point, different extent and impact of DNA methylation have been reported (e.g. (Witzmann *et al.*, 2012)).

In humans, the work of Richard Tremblay and Moshe Szyf have been focussing on early-life aggressive manifestations in boys exposed to adverse early environment and adult DNA methylation. Amongst others, they found altered DNA methylation at the *SLC6A4*/*5-HTT* promoter (Wang *et al.*, 2012), at cytokine loci and their respective transcription factors (Provençal *et al.*, 2013) as well as at numerous promoters of aggression-related genes (Provençal *et al.*, 2014) in this context. As another interesting candidate the Catechol-O- Methyltransferase (COMT) gene, which is related to diverse psychiatric disorders and aggression-related behaviours has been identified (Qayyum *et al.*, 2015). An interrogation of the methylation status of related CpG islands of the whole gene uncovered an association between DNA methylation and the non-synonymous Val158Met SNP, resulting in a functional amino acid substitution. Categorizing the cohort by this SNP, an association of DNA methylation and alcohol use was found. Moreover, DNA methylation at multiple COMT CpG sites was associated with socioeconomic status and ethnicity (Swift-Scanlan *et al.*, 2014). Interestingly, a Swedish cohort study was able to show an interaction of the Val158Met, with exposure to violence, parent-child relationship and physical aggression (Tuvblad *et al.*, 2016). Another important study in healthy adults focused on GR Exon 1_7 methylation in relation to life events and life time disorder. In this study, Tyrka et al. (2016) found that environmental adversities were negatively related to GR Exon 1_7 promoter methylation. This was dependent on the life history of mental disorder. Furthermore, past or present disorder as well as childhood adversity was associated with lower methylation levels. Next to adversity or disorder related factors, DNA methylation at the GR promoter was linked to altered cortisol responses to HPA axis challenge with dexamethasone. McGowan and colleagues were able to associate decreased levels of GR mRNA, in particular also of the splice variant containing exon 1F

with childhood abuse in the hippocampi of suicide victims (McGowan *et al.*, 2009). This decrease in expression was concomitant with increased cytosine methylation at the GR promoter and decreased nerve growth factor-induced protein A (NGFI-A)-binding. A study in healthy Caucasian males showed that life events had the capacity to alter DNA methylation at the *5-HTT* gene (Duman and Canli, 2015). The change in methylation was furthermore dependent on the 5-HTTLPR genotype. This change in methylation was accompanied by genotype specific *5-HTT* mRNA expression upon acute stress exposure. In macaques, a higher *5-HTT* gene methylation was observed in carriers of the short allele. This was independent of rearing conditions. Interestingly, *5-HTT* expression was independent of the genotype and only predicted by the methylation state of the gene. Similarly, behaviour was predicted only by an interaction of gene methylation and rearing conditions (Kinnally, Capitanio and Leibel, 2010).

Taken together, these observations in humans and non-human primates suggest a strong implication of epigenetic regulation, pointing out various candidate molecular players. However, one has to keep in mind that epigenetic mechanisms are also known to be critically involved in cell-differentiation and, as such represent tissue- and also cell-type specific markers, making the examination of environmental or genotypic effects on the epigenome incomparably more complex. For the lack of access to brain tissue in living patients, psychiatric research has to resort to readily available tissues, like the blood or saliva, consisting of numerous epigenetically profoundly distinct cell-types when compared to those observed in the brain. Several studies addressed this limitation by correlating the blood and brain methylome (Hannon *et al.*, 2015; Yu *et al.*, 2016). Such studies did not confirm a general predictive quality of blood-derived DNA methylation for assessment of the brain methylome status, but high inter-individual correlations between tissues, suggest that certain genetic variations might influence at least a subset of loci equally in diverse tissue. Thus, overall findings comparing tissues promote the idea of a more complex relationship between tissue-specific and general methylation patterns. To further investigate tissue-specific effects, animal models can provide a valuable tool in unravelling the relations between epigenetic marks and their role in disease. In rodent studies one mechanism of early-life programming of the HPA axis via the 5-HT system was suggested (Smythe, Rowe and Meaney, 1994; Meaney *et al.*, 2000; Laplante, Diorio and Meaney, 2002). Within the first week of postnatal life, the tactile stimulus of maternal licking and grooming was shown to increase 5-HT turnover in the hippocampus (Smythe, Rowe and Meaney, 1994), subsequently activating the 5-HT_7 receptor. In turn, 5-HT_7 receptor activation is thought to trigger a signalling cascade that regulates gene expression through numerous transcription factors, such as cAMP-response element binding protein (CREB), resulting in an increased expression of NGFI-A and CREB binding protein (CBP) (Meaney *et al.*, 2000). Finally, NGFI-A and CBP form a complex, binding to the exon 1_7 promoter sequence of the GR, altering the gene's methylation levels, eventually resulting in an increased GR expression in the hippocampus (Weaver *et al.*, 2004, 2007). Later findings revealed that the observed change in GR methylation levels may, at least partially, be accounted for by DNA hydroxymethylation. Next to the observed direct modifications at the DNA strand itself, accompanying histone modifications, associated with activated transcription, were found to correlate positively with methylation (Zhang *et al.*, 2013). In another animal model of adverse caregiving in rats, tissue from the PFC was found to comprise an increased methylation of the gene, encoding brain-derived neurotrophic factor (*Bdnf*) in adult animals that had experienced the experimental adverse caregiving conditions (Roth *et al.*, 2009). These animals also

had less *Bdnf* mRNA in the PFC. Subsequent work revealed DNA methylation (Roth *et al.*, 2014; Doherty, Forster and Roth, 2016) and histone acetylation alterations in additional brain regions, dependent on sex and cell-type (Blaze, Asok and Roth, 2015). Another recent study in rats showed a change in expression levels of the *Mao-a* gene upon stress exposure during adolescence, in a region specific manner (Márquez *et al.*, 2013). The observed changes in expression were concomitant with increased histone acetylation at the *Mao-a* gene. The molecular changes were furthermore accompanied by altered amygdala activity and connectivity as well as increased aggression. In another study in adult rats, chronic stress exposure had an effect on stress reactivity and on methylation levels at CpG clusters at the *Gr* gene in adrenal and pituitary gland (Witzmann *et al.*, 2012). Interestingly, no effect of chronic stress was observed in the PVN. In this region, the inter-individual variances in *Gr* 1_7 promoter methylation were higher than between conditions. This might indicate earlier programming in this region, supporting the hypothesis of a time-dependent window of epigenetic programming (Witzmann *et al.*, 2012) and suggests the importance of individuality even in highly controlled animal models. The concept of individuality, i.e. differential susceptibility, in animals has been discussed with regard to several behavioural manifestations, including coping behaviour and aggression and has to be considered even more relevant in the light of epigenetic programming (Benus, Koolhaas and Oortmerssen, 1987; de Boer, Vegt and Koolhaas, 2003; Koolhaas *et al.*, 2007).

Conclusion

In this review, we suggest that exaggerated, sociopathy-like aggression, related to early adversity, is mediated primarily by epigenetically-induced changes in amygdala-centred networks, modulated by reciprocal interactions between the HPA axis and the 5-HT system. As outlined in the course of this review, the classification of aggression subtypes represents a very important first step towards unravelling its origins. Notably, it seems that in case of abnormal aggression-related behaviours, the 5-HT system can serve as one distinctive regulator. Increased or decreased levels of 5-HT are leading to a shift in 5-HT signalling, which in turn interacts with functional networks and leads to aberrant behaviours.

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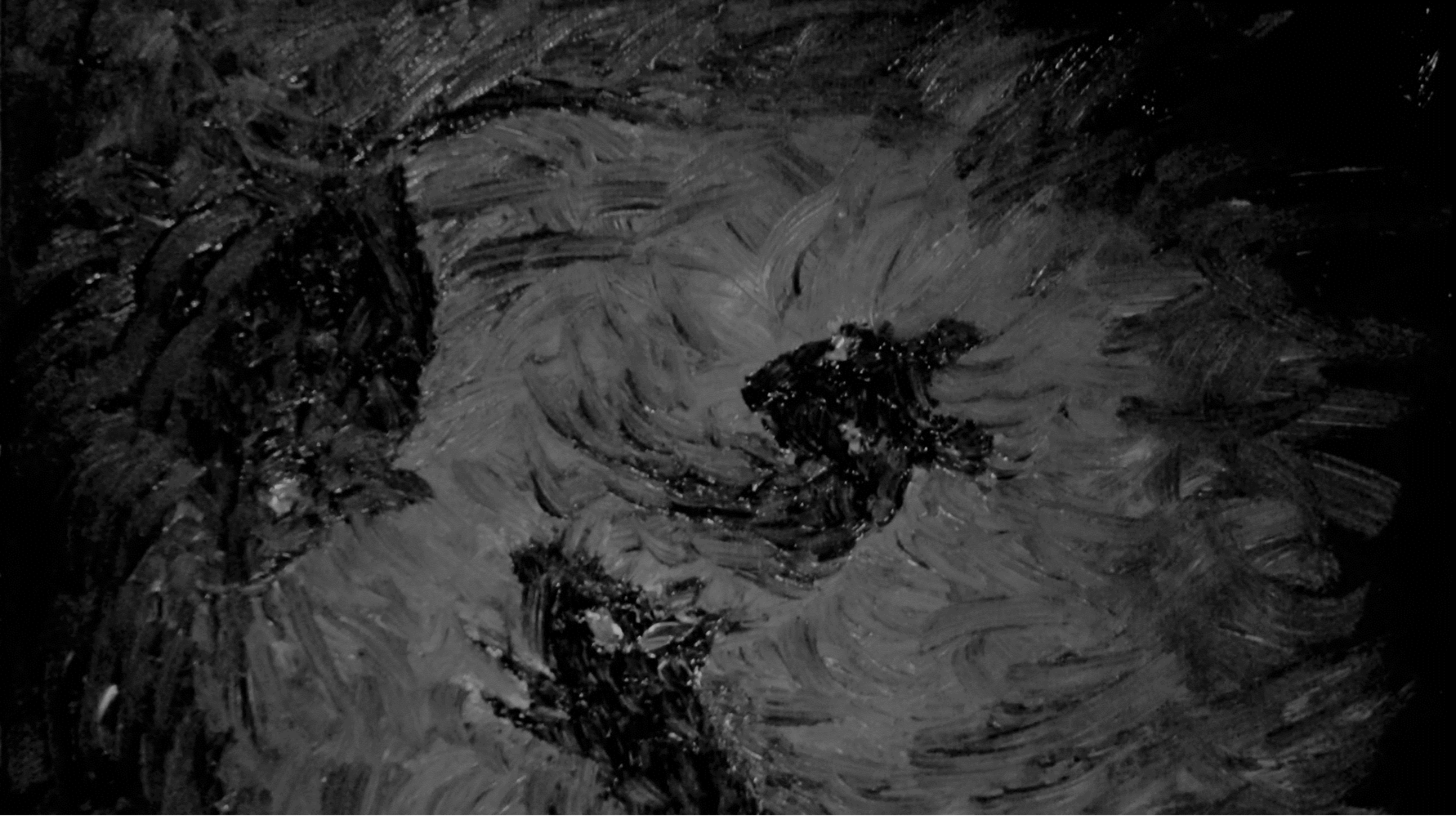

Impact of early-life stress on socio-emotional behaviour and the transcriptome and methylome within the amygdala of brain serotonin-deficient mice

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Abstract

Converging evidence suggests a role of serotonin (5-hydroxytryptamine, 5-HT) in modulating long-term neurobiological effects of early-life events. Here, we aimed at further elucidating the molecular mechanisms underlying this interaction, and its consequences for anxiety and social behaviours. To meet this end, adult, male tryptophan hydroxylase 2 null mutant (*Tph2*$^{-/-}$) and heterozygous (*Tph2*$^{+/-}$) mice, with a full or partial depletion of brain 5-HT, respectively, and their wildtype littermates (*Tph2*$^{+/+}$) were exposed to neonatal maternal separation (MS) and studied for behavioural and physiological changes, followed by RNA sequencing and methylome profiling. In general, full 5-HT depletion profoundly affected socio-emotional behaviours as well as hypothalamic-pituitary-adrenal (HPA) axis activity and body-weight in comparison to Tph2$^{+/+}$ littermates. *Tph2*$^{-/-}$ mice showed increased locomotor activity in a non-aversive, novel environment, decreased anxiety in an approach-avoidance conflict paradigm, decreased social behaviour as well as increased aggression, and a more pronounced stress response to behavioural testing relative to *Tph2*$^{+/+}$ littermates. By contrast, *Tph2*$^{+/-}$ offspring showed an ambiguous profile with task-dependent behavioural responses and unaltered physiology. While the observed effects regarding *Tph2* genotype were very pronounced, no clear MS effects were observed. However, MS was found to wash out some of the observed genotype-induced differences. On the molecular level, *Tph2* genotype and its interaction with MS differentially affected expression of numerous genes, of which a subset (3%) showed an overlap with DNA methylation profiles at corresponding loci. One gene with associated changes in methylation and expression was cholecystokinin, which was also found to be related to anxiety in the current study. Overall, full and partial depletion of brain 5-HT were related to pronounced behavioural changes, which, at least in part, were indicative of the observed effects of MS in *Tph2*$^{+/+}$ offspring, supporting the initially suggested role of 5-HT in early life programming.

Introduction

The serotonin (5-hydroxytryptamine; 5-HT) system is one of the brain's key neuromodulatory systems and, as such, involved in the manifestation of manifold behaviours (Baldwin and Rudge, 1995; Hariri and Holmes, 2006; Canli and Lesch, 2007). Mice, carrying a constitutive knock out of the tryptophan hydroxylase 2 (*Tph2*) gene (Gutknecht *et al.*, 2008), which encodes the rate-limiting enzyme of 5-HT synthesis in the brain (Gutknecht *et al.*, 2008; Alenina *et al.*, 2009; Mosienko *et al.*, 2012), were shown to be completely depleted of 5-HT in the brain. Moreover, mice of this line were found to display distinct behavioural profiles, concomitant with differential activity of various brain regions (Waider *et al.*, 2017). For example, male mice that were completely depleted of brain 5-HT showed increased flight behaviour upon exposure to novel environment as well as an exaggerated response to a footshock, administered during fear conditioning. Interestingly, the complete depletion of 5-HT was concomitant with a higher neural activity in the basolateral amygdala under home cage conditions, which was blocked upon exposure to the novel environment. Footshock-induced neural activity was comparable to the activity observed in wildtype littermates, while in mice with only partial depletion of the gene, home cage neural activation was unaltered when compared to wildtype mice but increased following footshock in comparison to the activity observed in fully depleted mice. Next to these observations, *Tph2*-deficient mice showed a distinct corticosterone profile in the context of a chronic mild stress paradigm and stress-induced hyperthermia (Gutknecht *et al.*, 2015). Next to the increased activity, fully brain 5-HT-depleted males were found to be more aggressive, when compared to their wildtype counterparts (Mosienko *et al.*, 2012).

Numerous studies in humans, non-human primates, and rodents, found genetic variations in genes, associated with the 5-HT system, to have an effect on emotion regulation. This was observed, in particular, in interaction with aversive experiences in early-life (Bennett *et al.*, 2002; Caspi *et al.*, 2002, 2003; Champoux *et al.*, 2002; van den Hove *et al.*, 2011; Sachs *et al.*, 2015; Wong *et al.*, 2015). Early-life adversity has the capacity to affect the stress response and stress-related behaviours later in life by reprogramming the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Thompson, 1957; Levine, 1967). Already throughout the prenatal period and further along throughout life, particularly limbic brain areas are sensitive to specific effects of adversity in early life and genetic predisposition (Lupien *et al.*, 2009). For example, over-reactivity of the amygdala has been associated with maladaptive emotional behaviours, including increased aggression and impulsivity and dampened prefrontal cortex (PFC) reactivity (Canli *et al.*, 2005; Pezawas *et al.*, 2005; Meyer-Lindenberg *et al.*, 2006; Márquez *et al.*, 2013). In particular the genetic makeup of the 5-HT system is suggested to play a role in the modification of limbic system function (Meyer-Lindenberg *et al.*, 2006; Márquez *et al.*, 2013). Moreover, 5-HT was found to be critically involved in mediating consequences of early-life stress on the molecular level. 5-HT signalling during the early postnatal period was shown to alter epigenetic modifications, e.g. by facilitating the recruitment of chromatin remodelling factors and transcription factors, such as cAMP-response-element-binding-protein-binding protein (CBP) and early growth response protein 1 (Egr1, also referred to as Ngfi-a) (Hellstrom *et al.*, 2012). Besides its function in epigenetic regulation, 5-HT system functioning during this period was suggested to be vital for the fine tuning of 5-HT projections in several limbic brain regions (Migliarini *et al.*, 2013). Furthermore, early stress, such as maternal separation (MS), was shown to influence the expression of 5-HT-related

factors. For example, exposure to MS was associated with increased monoamine oxidase a (*Mao-a*) expression in striatum and brain stem (Wong *et al.*, 2015), decreased 5-HT immunoreactivity in the hypothalamus (Veenema *et al.*, 2006) as well as increased *Tph2* expression in the dorsal raphe (DR), in the context of social challenges (K. L. Gardner *et al.*, 2009; Katherine L. Gardner *et al.*, 2009).

In the present study, we aimed to further elucidate the role brain 5-HT plays in the epigenetic mediation of early, postnatal adversity and the effect this interaction exerts on aggression and anxiety-related behaviours. To this end, *Tph2* null mutant (*Tph2*$^{-/-}$) pups as well as their wildtype (*Tph2*$^{+/+}$), and heterozygous (*Tph2*$^{+/-}$) littermates were exposed to a maternal separation (MS) paradigm during the early postnatal period. Adult, male offspring of both non-stressed, control and MS litters were subjected to behavioural screening and gene expression as well as DNA methylation profiling.

Materials and methods

Animals and procedures

All experiments were performed in accordance with the European Parliament and Council Directive (2010/63/EU) and approved by local authorities (Würzburg: 55.2-2531.01- 57/12). All efforts were made to minimize animal numbers and suffering of the animals.

Mice of the parental generation (breeding facility, ZEMM, Würzburg), of either sex, were housed in sex-specific groups of 2-7 under 14 h/10 h light-dark cycle, with lights on at 7AM - 9PM, in climate-controlled rooms (21±1°C, humidity 45-55%). Standard rodent chow and water were available *ad libitum*. For breeding the test generation, one male and two female *Tph2*$^{+/-}$ mice, fully backcrossed onto C57BL/6N genetic background and approximately 3 months of age, were put together in standard (267 x 207 x 140 mm) polysulfone cages (Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany) with woodchips and standard nesting material. Females were tested for vaginal plugs twice a day. All mating pairs were separated after 5 days and females that had shown a plug at least once were housed individually from then onwards. Females were weighed before mating and 4, 7 and 10 days after separation. Animals that did not show any weight gain over the first 10 days after separation were mated again, with different males. From 14 days after separation of the breeding pairs, nests were checked for pups once a day. The day of birth was declared P0 and no cage changes were performed until P5. At P2, litters were randomly assigned to MS and control group.

MS was performed according to a protocol adapted from Veenema and colleagues (Veenema *et al.*, 2006). In brief, from P2 – P15, MS litters and their respective nesting material, were removed from the dam's cage and brought into an adjacent room, where they were kept under a heating-lamp at humidity levels of above 60% for 3 h each day. The separation time point was randomly assigned during the light interval. The control litters were left undisturbed in their home cages except for P5, P10 and P15, when litters and dams, of both the control and MS group, were weighted and P5, P12 and P19, when cages were changed. Pups were weaned at P24±2. For all following procedures, exclusively male offspring were used. After weaning mice were housed individually in standard cages under a 12 h light-dark cycle, with lights on at 1AM – 1PM. Animals were weighed at P24±2, P45±2 and 68±4 during routine cage changes. Behavioural testing started at around P56 and all tests were conducted during

the dark phase between 2PM and 6PM. Each animal was allowed to rest for at least 6 days in-between the different behavioural tests and for two weeks after the last test, before being sacrificed at around P115 using isoflurane, followed by decapitation. Brains were harvested within 2 min of decapitation and carefully frozen in isopentane at -80°C.

Behavioural testing

Behavioural tests were performed in the following order, starting with the dark-light box (DLB) test, followed by open-field (OF) test and elevated plus maze (EPM) and as last test the repeated resident-intruder test (RIT). The order, in which animals were tested, was randomised over genotype and treatment group. For all tests, unless stated otherwise, mice were tracked using infrared light from below the respective apparatus. Trials were recorded from above, using an infrared-sensitive camera. Later on, behavioural analysis was performed using VideoMot2 tracking software (TSE Systems, Bad Homburg, Germany). In-between trials, the respective apparatus was cleaned with Terralin liquid (Schülke, Norderstedt, Germany).

The DLB test is an approach-avoidance conflict paradigm and was used to determine the innate level of avoidance behaviour (Crawley and Goodwin, 1980; Onaivi and Martin, 1989). The DLB apparatus is an arena made from opaque, white, acrylic glass with an edge length of 50 cm. This arena is divided into a light compartment (34 cm x 50 cm x 40 cm) with on average 115 lux in the centre and 80 lux in the corners and a dark compartment (16 cm x 50 cm x 40 cm) with 0.1 lux. The two compartments are connected through an exit hole and animals can move freely between compartments. Each tested animal was placed into the dark compartment, facing the front right corner, and was allowed to explore the arena freely for 5 min (Waider *et al.*, 2017). Subsequently, distance moved and time spent in the dark and in the light compartment as well as the latency to enter the light compartment and the total distance moved were analysed.

The OF test was used to determine exploratory behaviour and locomotion in a novel, environment. The OF apparatus is an open arena made from black, acrylic glass with an edge length of 50 cm and a wall height of 40 cm. The test was performed under red-light (Post *et al.*, 2011). The tested animal was placed into the arena facing the front right corner and was allowed to explore the arena freely for 20 min. Subsequently, distance and time in the centre and global distance moved were analysed.

The EPM test is a conflict anxiety test, investigating avoidance behaviours, similar to the DLB test, but with the additional aversive stimulus of height. The EPM apparatus is a plus-shaped acrylic glass construct (TSE Systems, Inc., Bad Homburg, Germany) made from black opaque PERSPEX XT, semi-permeable to infrared light, with two opposing closed arms (30 cm × 5 cm), comprised by 15 cm high walls, and two opposing open arms without walls (30 cm × 5 cm, with 0.5 cm wide boundaries elevated 0.2 cm). The four arms meet in the centre to form a square of 5 cm × 5 cm. The maze was raised 62.5 cm above the ground. On the open arms, the brightness measured was on average 70 lux, in the centre 20 lux, and in the closed arms 1 lux. Each animal was placed in the centre facing an open arm and allowed to explore the maze for 5 min (Post *et al.*, 2011; Gutknecht *et al.*, 2015). Subsequently, distance and time on the open arms, in the closed arms and the centre, as well as the number of entries onto the open arms were analysed (Lister, 1987).

The RIT is an aggression test based on the naturally occurring territoriality, observed in male mice. The used protocol was adapted from the Koolhaas group in Groningen (Koolhaas *et al.*, 2013). In preparation of the test, in order to keep olfactory cues stable and thus reinforce natural territoriality in the residents, a small amount of soiled sawdust was carried over with every cage change. From 5 days prior to testing until one day after testing, cages were not changed. Intruder males were approximately 2 months old DBA2/N males (Charles River, Sulzfeld, Germany). They were housed in groups of 6 under a 14 h/10 h light-dark cycle with lights on at 7AM - 9PM, in climate-controlled rooms (21±1°C, humidity 45-55%) and were allowed to adapt for at least 20 days before testing started. Standard rodent chow and water were available *ad libitum*. All intruders were weighed prior to the RIT to allow weight matching them to a respective resident. The heaviest intruder per cage was excluded from testing and used prior to the first RIT for instigation. Each instigation lasted 5 min, allowing to stimulate residents by visual and olfactory exposure to an intruder without the possibility of physical contact (de Almeida and Miczek, 2002; de Almeida *et al.*, 2005). 3 min after instigation the actual intruder was introduced into the resident's home cage. The RIT was performed under red-light and repeated for 4 times, with 24 h intervals between each encounter. For each encounter the resident was matched with an unfamiliar intruder. The test time added up to 5 min from the first attack bite (as observed online) or maximum 10 min. Encounters were filmed from a side-on view for subsequent behavioural analysis. A red-light sensitive high-speed camera (The Imaging Source, Bremen, Germany) was used, recording at 120 fps. Following each encounter, resident and intruder were examined for wounds. Recordings of the first as well as the fourth (last) encounter were analysed in detail for all animals that were further screened for whole genome RNA expression and DNA methylation (n = 8-10 per group) using Observer XT software (Noldus, Wageningen, The Netherlands). Scored behaviours were categorised in 5 groups, i.e. non-social (rearing, digging, self-grooming and walking); social (sniffing, grooming, turning to, following, and looking towards), dominant (grooming, mounting, and following, while the intruder showed clear signs of submission), threat (tail rattling, sideway threatening, up-right posturing, boxing, aggressive grooming and fast following) and aggression (clinching, keeping down, biting, kicking and chasing). One *Tph2*$^{+/-}$ offspring of the control group had to be excluded from the analysis for the fourth session based on erratic behaviour of the intruder that did not allow for a representative evaluation of resident behaviour and one *Tph2*$^{+/+}$ offspring of the control group had to be excluded from the first session due to technical issues with the video.

Measurement of feacal corticosterone metabolites

To investigate HPA axis functionality in a non-invasive manner, faecal boli, accumulated over 7 days, were collected during the weekly cage change. Feacal samples were taken at three time-points throughout the experimental timeline: once before behavioural testing, once, one day after EPM exposure and a last time at the day of sacrifice. Following sample collection, feacal boli were stored at -20°C, until further processing. Feacal cortsicosterone metabolites (FCMs) were extracted (50 mg powdered faeces plus 1 ml 80% methanol) and, subsequently, measured with a 5α-pregnane-3ß,11ß,21-triol-20-one enzyme immunoassay (EIA). This assay was developed and successfully validated for mice. For description see (Touma *et al.*, 2003; Touma, Palme and Sachser, 2004), where also cross-reactions with other steroids are described in detail. To account for plate differences, 2

samples of each group and time point were assigned per plate and each value was calculated relative to the complementary baseline, wildtype, control samples per plate and reported in %.

Extraction and sequencing of nucleic acids

For the whole genome approaches, both DNA and RNA were extracted from amygdala tissue. Frozen brains were semi-thawed on a cooling plate (-6°C) and the amygdala was rapidly dissected using a stereo microscope (Olympus, Hamburg, Germany). Before extracting nucleic acids, the tissue of left and right amygdala was powderised at -80°C, blended and split into two homogenous portions, of which one portion was used to investigate RNA expression, the other to investigate DNA methylation.

Total RNA sequencing

Extraction of RNA was performed, using a combination of the classic phenol-chloroform method and the column-based protocol of the commercial miRNeasy Mini kit (Qiagen, Hilden, Germany) for fatty tissue. The frozen samples were homogenized in QIAzol lysis reagent with a precooled stainless steel bead (Qiagen, Hilden, Germany), using the TissueLyzer (Qiagen, Hilden, Germany) at 20 Hz and 4°C, for 60 s. Subsequently, homogenates were incubated for 5 min at room temperature (RT), mixed with 60 µl of chloroform (Roth, Karlsruhe, Germany) and incubated for another 10 min on ice before the aqueous phase was recovered by phase separation and mixed with 1.5 volumes ethanol (95-100%). Then samples were transferred to miRNeasy Mini columns and consecutively washed using the kit-specific RWT and RPE buffers. To minimise the risk for genomic DNA contamination the samples were incubated with DNase (Qiagen, Hilden, Germany) in-between RWT buffer washes, according to kit instructions. RNA was finally eluted in RNase free H_2O provided with the kit and stored at -80°C. RNA quality was assessed before further processing using the Experion capillary electrophoresis (Biorad, München, Germany) according to the manufacturer's instructions. Only samples with Experion RNA quality indicator (RQI) values between 8.0 and 9.8 were considered for further analysis. In addition, RNA concentrations were determined with the Nanodrop (Thermo Scientific, Wilmington, Delaware, USA).

Following extraction, samples were brought to a concentration of 50 ng/µl and shipped to IGA Technologies (Udine, Italy) for further processing. In brief, library preparation was performed using the TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, US) that captures coding RNA and multiple forms of non-coding RNA, with a total input of 500 ng of RNA. The sequencing was performed using the Illumina HiSeq 2500 platform at a read-length of 125 bp with paired-end reads, yielding 60 million reads/sample. Follow up data demultiplexing, clean up and quality control was performed by IGA Technologies. Mapping was performed by the Core Unit Systems Medicine at the University of Würzburg, where reads were mapped to the *mus musculus* GRCm38.p5 genome using STAR (Dobin *et al.*, 2013). Subsequently, the reads per position were determined using HTSeq (Anders, Pyl and Huber, 2015) resulting in count-tables.

DNA methylation-based enrichment sequencing

Extraction of genomic DNA was performed using a standard phenol/chloroform/isoamyl alcohol extraction protocol. The frozen samples were homogenized in 0.5 % SDS extraction buffer with a

precooled stainless-steel bead (Qiagen, Hilden, Germany) using the TissueLyzer (Qiagen, Hilden, Germany) at 20 Hz and 4°C, for 60 s. Subsequently, samples were incubated with 500 µg proteinase K (Applichem, Darmstadt, Germany) at 55°C for at least 3 h followed by 1 h incubation with 500 µg RNase A (Roche, Basel, Switzerland) at 37 °C. Subsequent DNA isolation through phase separation and DNA precipitation from the aqueous phase were performed as described elsewhere (Schraut *et al.*, 2014). DNA was stored at -80°C for further use. DNA concentrations were determined using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA USA) with the Qubit high sensitivity kit for double-stranded DNA (Thermo Fisher Scientific, Waltham, MA USA).

Following extraction, all samples were brought to a concentration of 11 ng/µl and shipped to Nxt-Dx (Ghent, Belgium) for further processing. Briefly, 300 ng of the extracted DNA were sheared by sonication followed by DNA capture using methyl binding domain (MBD) of human methyl-CpG binding protein 2 (MeCP2). Subsequently, library preparation was performed followed by multiplexed paired-end sequencing with a read-length of 50 bp and an approximate output of 20 million reads/sample on the Illumina HiSeq4000 platform. Following, data demultiplexing, clean up and quality control, reads were mapped to the *mus musculus* GRCm38.p5 genome using Bowtie2 (v2.1.0) software in “end-to-end & sensitive-mode”. After mapping, coverage peaks were generated using MACS 14 peak caller (Zhang *et al.*, 2008). Subsequently, peaks were aligned and sequencing reads within overlapping peak sets were counted using the DiffBind R-package v2.0.9 (Stark and Brown, 2013). This resulted in count-tables of the methylated loci. Genes were annotated based on the first nearest feature, using ChIPpeakAnno (3.8.9) (Zhu *et al.*, 2010) with TxDb.Mmusculus.UCSC.mm10.ensGene: Annotation package for TxDb object(s), R package (version 3.4.0.; 2016) and EnsDb.Mmusculus.v75 Ensembl based annotation package, R package (version 2.1.0; Maintainer: Johannes Rainer 2016).

Statistical analysis

For statistical analysis of behaviour and physiological measures SPSS Statistics (IBM Deutschland GmbH, Ehningen, Germany) was used. Data was examined for normal distribution and outliers, using the Shapiro-Wilk test and boxplots. As a considerable number of factors did not meet assumptions for parametrical testing, Kruskal-Wallis test, investigating the factors *Tph2* genotype, MS and experimental group (as determined by the combination of *Tph2* genotype and MS), was performed to test for main effects or combined main effects. Main effects were followed up by Mann-Whitney U test. Due to the nature of the FCM samples repeated measures multifactorial ANOVA was employed (time, *Tph2* genotype and MS). Main effects of the repeated measures ANOVA were investigated using student’s t test (Bonferroni corrected). Statistical correlations were calculated using Spearman’s correlation coefficients. P-values < 0.05 were considered significant.

For statistical analysis of RNA expression and DNA methylation in the amygdala, the DESeq2 R-package (Love, Huber and Anders, 2014) was used and comparisons were made, dependent on developmental stress in the context of diverse levels of brain 5-HT depletion in $Tph2^{+/+}$, $Tph2^{+/-}$ and $Tph2^{-/-}$ mice based on the count tables. Contrasts were calculated for genotype G1 [($Tph2^{+/-}$ MS + $Tph2^{+/-}$ C) - ($Tph2^{+/+}$ MS + $Tph2^{+/+}$ C)] and G2 [($Tph2^{-/-}$ MS + $Tph2^{-/-}$ C) - ($Tph2^{+/+}$ MS + $Tph2^{+/+}$ C)] as well as gene-by-environment (GxE) interactions GE1 [($Tph2^{+/-}$ MS - $Tph2^{+/-}$ C) - ($Tph2^{+/+}$ MS - $Tph2^{+/+}$ C)] and GE2 [($Tph2^{-/-}$ MS - $Tph2^{-/-}$ C) - ($Tph2^{+/+}$ MS - $Tph2^{+/+}$ C)] for both analyses. The effect directions

of MS, stratified per genotype E1 (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C), E2 (*Tph2* $^{+/-}$ MS - *Tph2* $^{+/-}$ C) and E3 (*Tph2* $^{-/-}$ MS - *Tph2* $^{-/-}$ C) are displayed in S 5. For the RNA sequencing experiment, this resulted in lists of differentially expressed genes (DEGs), comprising the base mean of all counts per gene, the ratio on a log2 scale (log2 Fold Change; lg2FC), the log fold change standard error, the Wald statistic, p-value and adjusted p-value. For the RNA analysis, only genes with a base mean > 0 were taken into account for the analysis and one gene (i.e. *Lars2*) was excluded due to an incomparably high base mean. A similar output was obtained for the analysis of the differentially methylated loci (DMLs), comprising, in addition to the statistics results and peak locus, a list of associated genes and their features. Pre-analysis quality control revealed two outlier samples, both of the *Tph2*$^{-/-}$ MS group, with remarkably lower amount of counts, shifted normalised density and additional small peaks as well as an overall shifted peak in the density histogram of signal intensities (for quality control summary see S6 and S7). Genes of either analysis were determined differential if they showed a nominal p-value < 0.01 and lg2FC > |0.2|. Finally, the overlap of DEGs and DML-associated genes per comparison was investigated and non-parametrical, linear regression analysis was performed on the raw counts of overlapping RNA and MBD sequencing genes, as well as behaviour and FCM levels using the Spearman correlation coefficient (ρ).

In addition, pathway enrichment analysis was conducted using the pathway analysis tool PathVisio (van Iersel *et al.*, 2008; Kutmon *et al.*, 2015), which operates based on the wikipathways platform (Kelder *et al.*, 2012; Kutmon *et al.*, 2016). Gene ontology (GO)-term analysis was conducted using the online tool EnrichR (Chen *et al.*, 2013; Kuleshov *et al.*, 2016). For the analysis, the output of both RNA and MBD sequencing were sorted based on the p-value, from lowest to highest p-value. Unique gene names annotated to the top 500 genes/loci of these lists were used as test lists. Of the top 10 pathways or GO-terms, only pathways with a z-score > 2 or GO-terms with a p < 0.05 and an enrichment of more than 3 genes were considered as enriched.

Results

Dam and offspring physiological parameters

MS had no effect on dam or litter weight and pre-weaning offspring survival, as depicted in S 1 Table. Post-weaning, a genotype effect on body-weight was observed in the offspring (P24 ($\chi^2(2) = 39.5$, $p < 0.001$); P45 ($\chi^2(2) = 28.0$, $p < 0.001$); P68 ($\chi^2(2) = 18.1$, $p < 0.001$)), with *Tph2*$^{-/-}$ offspring weighing less across the life span compared to *Tph2*$^{+/+}$ ($p < 0.001$) and *Tph2*$^{+/-}$ ($p < 0.001$) offspring, independent of MS exposure (Table1).

Table 1 Body-weight of control offspring or offspring, exposed to maternal separation throughout life (postnatal day 24–68).

	P24±2	P45±2	P68±4
control *Tph2*$^{+/+}$	14.0± 0.6	24.1± 0.6	27.3± 0.6
MS *Tph2*$^{+/+}$	15.0± 0.5	24.3± 0.6	27.3± 0.6
***Tph2*$^{+/+}$**	14.4± 0.4	24.2± 0.4	27.3± 0.4
control *Tph2*$^{+/-}$	14.7± 0.4	24.2± 0.5	27.3± 0.6
MS *Tph2*$^{+/-}$	14.2± 0.5	24.5± 0.4	27.4± 0.5
***Tph2*$^{+/-}$**	14.5± 0.3	24.3± 0.3	27.3± 0.4
control *Tph2*$^{-/-}$	8.6± 0.7	21.2± 0.6	24.9± 0.5
MS *Tph2*$^{-/-}$	9.1± 0.5	20.9± 0.6	24.7± 0.5
***Tph2*$^{-/-}$**	8.9± 0.4	21.1± 0.4	24.8± 0.3

Full depletion of tryptophan hydroxylase 2 (*Tph2*) decreases body-weight [g] when compared to *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ offspring, independent of MS ($p < 0.001$). Values represent group means ± standard errors (n = 8-18). *$p < 0.050$ (Post hoc: Mann-Whitney U).

Levels of FCM, over three time-points, revealed a genotype effect on FCM levels over time ($F(2.576, 79.85) = 5.4$, $p = 0.003$; Greenhouse-Geisser), independent of MS. Baseline FCM in *Tph2*$^{-/-}$ mice was higher when compared to *Tph2*$^{+/+}$ offspring ($p = 0.029$). Furthermore, only *Tph2*$^{-/-}$ animals showed a notable decrease following behavioural testing (test: $p = 0.003$, recovery: $p = 0.001$) with no observable difference between testing and recovery levels (Table 2).

Table 2 Metabolic corticosterone from faecal samples over three tim-points: baseline, following behavioural testing, recovery.

	baseline	testing	recovery
control *Tph2*$^{+/+}$	0.00 ± 0.09	-0.17 ± 0.13	-0.02 ± 0.28
MS *Tph2*$^{+/+}$	0.12 ± 0.28	-0.22 ± 0.13	-0.11 ± 0.16
***Tph2*$^{+/+}$**	**0.06** ± 0.14	-0.19 ± 0.09	-0.06 ± 0.16
control *Tph2*$^{+/-}$	0.01 ± 0.16	-0.37 ± 0.09	-0.33 ± 0.09
MS *Tph2*$^{+/-}$	0.01 ± 0.15	-0.16 ± 0.19	-0.22 ± 0.17
***Tph2*$^{+/-}$**	0.01 ± 0.11	-0.27 ± 0.11	-0.27 ± 0.09
control *Tph2*$^{-/-}$	0.51 ± 0.20	-0.24 ± 0.14	-0.35 ± 0.08
MS *Tph2*$^{-/-}$	0.48 ± 0.23	-0.49 ± 0.10	-0.49 ± 0.09
****Tph2*$^{-/-}$**	**0.49** ± 0.16	**-0.39** ± 0.08	**-0.44** ± 0.06

Full depletion of tryptophan hydroxylase 2 (*Tph2*) increased the baseline metabolic corticosterone (FCM) [%, calculation described in methods section] when compared to *Tph2*$^{+/+}$ offspring ($p = 0.029$) and altered the observed reactivity to stress ($p < 0.001$), independent of maternal separation (MS). Values represent group means ± standard errors (n = 8-18). *$p < 0.050$ (Post hoc: t-test, Bonferroni).

General exploratory activity and anxiety

In the OF, we observed an effect over all groups for the distance covered ($\chi^2(5) = 12.8$, $p = 0.026$), with *Tph2*$^{+/+}$ ($U = 33.0$, $p = 0.041$) and *Tph2*$^{+/-}$ ($U = 35.0$, $p = 0.040$) control offspring covering less distance when compared to *Tph2*$^{-/-}$ control offspring (Fig 1 A). In MS offspring this effect did not reach significance. Furthermore, *Tph2* genotype influenced the time spent ($\chi^2(2) = 8.0$, $p = 0.018$) and distance covered ($\chi^2(2) = 7.4$, $p = 0.025$) in the centre of the arena, independent of MS. *Tph2*$^{+/-}$ mice were covering a greater distance in the centre when compared to *Tph2*$^{+/+}$ mice ($U = 278.0$, $p = 0.007$), and spent more time in the centre when compared to *Tph2*$^{+/+}$ ($U = 319.0$, $p = 0.035$) and *Tph2*$^{-/-}$ mice ($U = 175.0$, $p = 0.009$).

In the DLB, no significant effects of *Tph2* genotype or MS were found. All animals showed a comparable latency to enter the light compartment and time spent (Fig 1B) as well as distance covered in the respective compartments and in the whole arena.

In the EPM, an effect over all groups was observed for time spent on the open arms of the maze ($\chi^2(5) = 19.9$, $p = 0.001$) and in the closed arms ($\chi^2(5) = 11.5$, $p = 0.042$; Fig 1 C). Independent of MS, *Tph2*$^{-/-}$ offspring spent more time on the open arms than *Tph2*$^{+/-}$ offspring, (C: $U = 31.0$, $p = 0.023$; MS: $U = 21.0$, $p = 0.002$). When compared to *Tph2*$^{+/+}$ offspring this effect only reached significance in offspring of the MS group ($U = 9.0$, $p < 0.001$). Furthermore, *Tph2*$^{-/-}$ MS offspring spent less time in the closed arms when compared to *Tph2*$^{+/-}$ ($U = 37.0$, $p = 0.026$) and *Tph2*$^{+/+}$ ($U = 16.0$, $p = 0.001$) MS offspring. Moreover, distance covered in the open arms was influenced by *Tph2* genotype, independent of MS ($\chi^2(5) = 26.6$, $p < 0.001$). Full *Tph2* depletion increased the distance covered on the open arm, when compared to *Tph2*$^{+/-}$ (C: $U = 14.0$, $p = 0.001$, MS: $U = 24.0$, $p = 0.003$) and *Tph2*$^{+/+}$ (C: $U = 19.0$, $p = 0.004$, MS: $U = 21.0$, $p = 0.002$) offspring. The total distance covered was not affected by *Tph2* genotype or MS. Also, the entries into the open arms were comparable between groups and genotypes. Off note, while the time in the centre was not affected by any of the investigated factors, the distance covered in the centre was increased in MS offspring, when compared to controls ($U = 599.0$, $p = 0.039$). In addition, time spent in the open arms correlated positively with the baseline FCM measures ($\rho = 0.261$, $p = 0.032$). Levels of FCM after the EPM and after a longer period of recovery correlated positively with time in the closed arms (stress: $\rho = 0.292$, $p = 0.016$; recovery: $\rho = 0.392$, $p = 0.001$) and negatively with time on the open arms (stress: $\rho = -0.276$, $p = 0.023$, recovery: $\rho = -0.370$, $p = 0.002$).

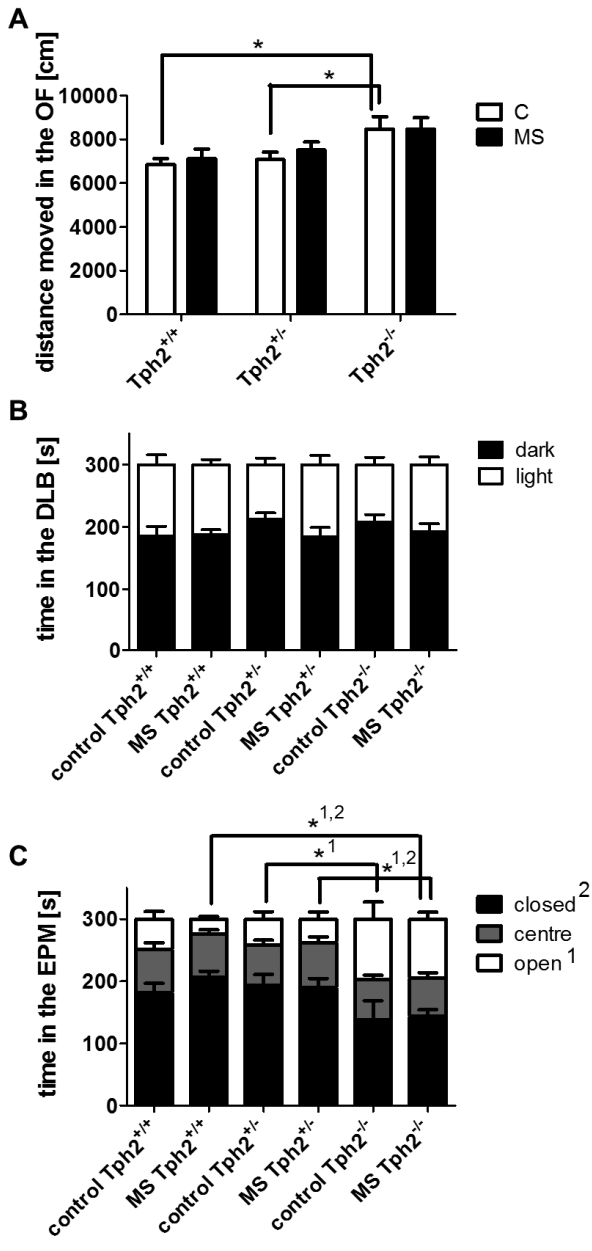

Figure 1 General activity and anxiety-related behaviours. (A) Open-field activity: full tryptophan hydroxylase 2 (*Tph2*) depletion increased the distance covered [cm] in control offspring compared to *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ control offspring ($p < 0.050$). Anxiety-related behaviours in (B) the dark-light box (DLB) and (C) the elevated plus maze (EPM): full TPH2 depletion affected the time [s] spend on either arm of the EPM, dependent on neonatal maternal separation (MS) exposure ($p < 0.030$). DLB performance was unaffected by both *Tph2* genotype and MS. Bars represent group means ± standard errors (n = 8-18). *p < 0.050 (Post hoc: Mann-Whitney U).

Social behaviours and aggression

In the first session of the repeated-resident intruder test (RIT), *Tph2* genotype was found to affect attack latency, independent of MS ($\chi^2(2) = 7.0$, $p = 0.031$). Full *Tph2* deficiency decreased the latency to the first attack when compared to *Tph2*$^{+/+}$ offspring ($U = 65.0$, $p = 0.011$; Fig 2). Moreover, the number of total attacks was affected by the *Tph2* genotype ($\chi^2(2) = 6.8$, $p = 0.033$), with *Tph2*$^{-/-}$ ($U = 76.0$, $p = 0.015$) and *Tph2*$^{+/-}$ ($U = 96.5$, $p = 0.032$) offspring attacking more often, when compared to*Tph2*$^{+/+}$ offspring (Fig 2). Moreover, *Tph2* deficiency was found to affect social behaviour ($\chi^2(2) = 9.4$, $p = 0.009$), dominance ($\chi^2(2) = 7.0$, $p = 0.030$) and threat ($\chi^2(2) = 9.3$, $p = 0.010$). Full depletion of *Tph2* increased the displays of dominance ($U = 70.0$, $p = 0.010$) and threat ($U = 62.5$, $p = 0.004$) and decreased social behaviours ($U = 59.0$, $p = 0.003$) compared to *Tph2*$^{+/+}$ offspring. Furthermore, also *Tph2* $^{+/-}$ offspring displayed increased threat behaviour when compared to *Tph2*$^{+/+}$ offspring ($U = 95.5$, $p = 0.031$; Fig 2). In addition, we found a negative correlation of baseline FCM with the relative time

spent on social behaviour (ρ = -0.387, p = 0.004) and a positive correlation with the relative time spent on non-social exploration of the environment (ρ = 0.275, p = 0.046).

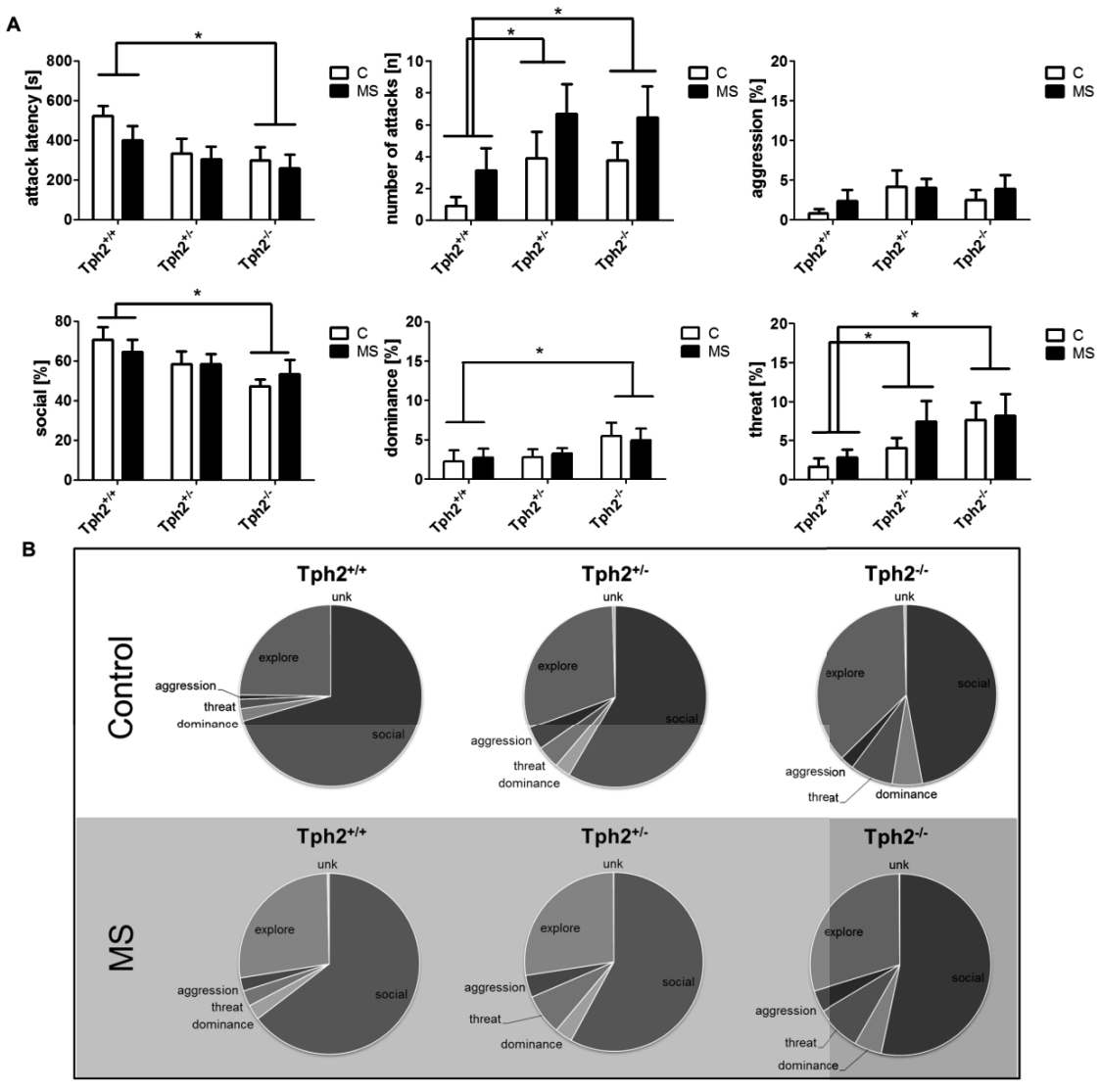

Figure 2 Behaviours during the first session of the repeated resident intruder test. A) Parameters, related to social and aggressive behaviour. tryptophan hydroxylase 2 (*Tph2*) depletion affected the display [%] of social, threat and dominant behaviours as well as the latency to attack [s] and the total number of attacks ($p < 0.050$), independent of maternal separation (MS). Bars represent group means ± standard errors (n = 8-10). *: $p < 0.050$ (Post hoc: Mann-Whitney U). B) Pie-chart overview of all the behaviours measured during the encounter. The displayed values represent group means.

During the fourth session of the repeated RIT, which represented the final repetition of the test, the difference between genotypes regarding attack latency no longer reached significance. The number of attacks was affected by *Tph2* genotype, independent of MS ($\chi^2(2) = 6.2$, p = 0.046) and was higher in *Tph2*-deficient offspring when compared to *Tph2*$^{+/+}$ animals (both *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$: U = 94.5, p = 0.032; Fig 3). An effect over all groups on aggression was observed ($\chi^2(5) = 12.3$, p = 0.031). Post hoc analysis revealed that *Tph2*$^{+/+}$ control animals spent less time attacking the intruder when compared to *Tph2*$^{-/-}$ control (U = 7.0, p = 0.005) offspring (Fig 3). Social behaviour was affected by *Tph2* deficiency ($\chi^2(2) = 8.1$, p = 0.017), with less relative time spent in social interaction in *Tph2*$^{-/-}$ offspring compared to *Tph2*$^{+/+}$ offspring (U = 72.0, p = 0.004; Fig 3). Threat behaviour was affected in a contrasting fashion by *Tph2* genotype ($\chi^2(2) = 6.0$, p = 0.050), with an increase in threat displays in *Tph2*$^{-/-}$ offspring,

compared to *Tph2*$^{+/+}$ offspring (U = 86.0, p = 0.016). Of note, recovery FCM was associated with the relative time spent on social behaviour (ρ = 0.286, p = 0.036) and the attack latency (ρ = 0.313, p = 0.023).

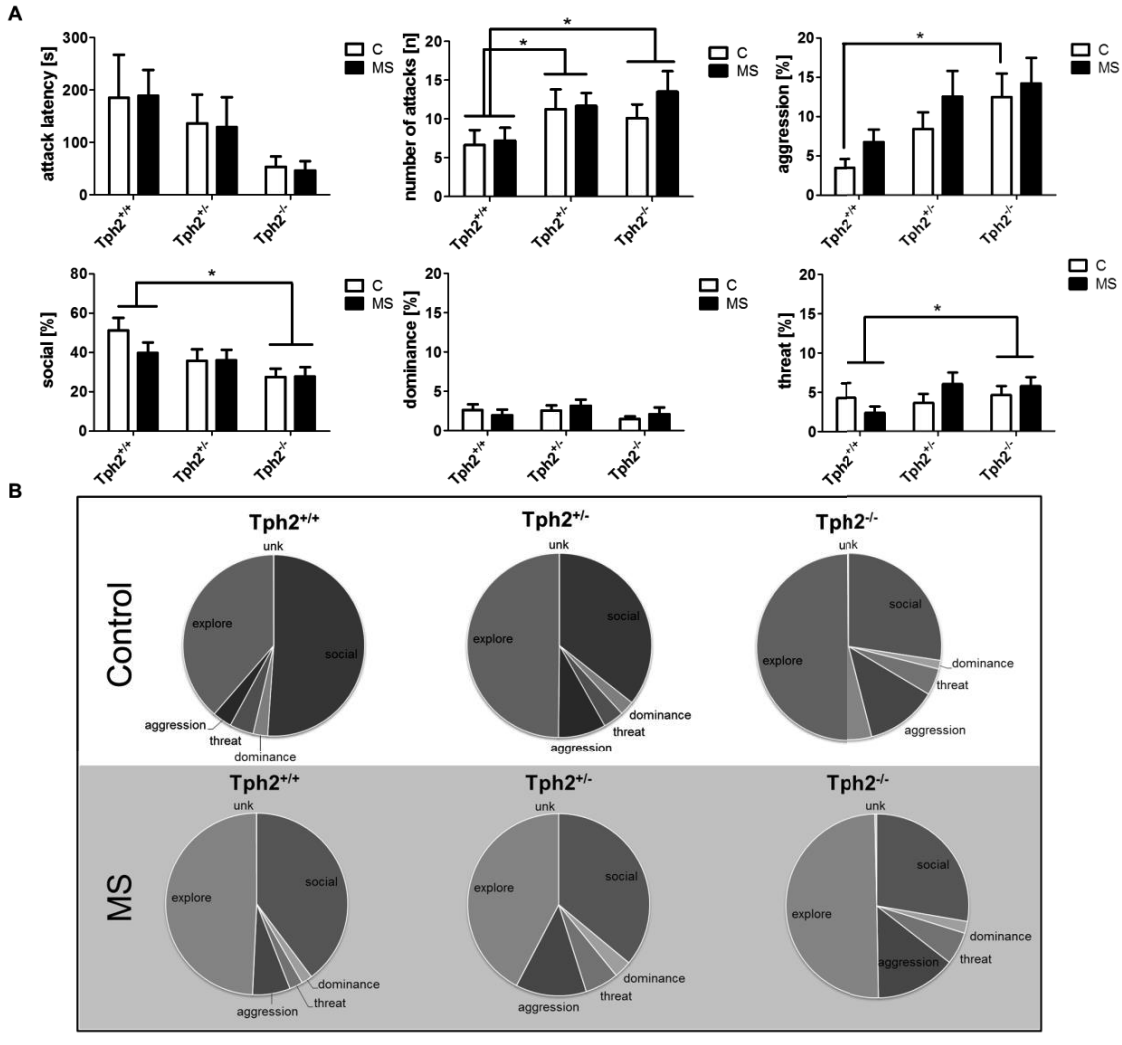

Figure 3 Behaviours during the fourth and final session of the repeated resident intruder test. A) Parameters, related to social and aggressive behaviour. tryptophan hydroxylase 2 (*Tph2*) depletion affected the display [%] of social and threat behaviours as well as the total number of attacks (p < 0.050), independent of maternal separation (MS). The displays of aggression [%] were affected in a *Tph2* genotype- and MS-dependent manner (p < 0.010). Bars represent group means ± standard errors (n = 8-10). *·p < 0.050 (Post hoc: Mann-Whitney U). B) Pie-chart overview of all the behaviours measured during the encounter. The displayed values represent group means.

Gene expression

Gene expression profiles were shown to be affected by *Tph2* genotype and its interaction with MS (Fig 4). The top 10 DEGs for each contrast are depicted in S 2 Table. Partial TPH2 depletion was associated with 96 DEGs. Of these, 54 (56.3 %) were down-regulated and 42 (43.7 %) were up-regulated in *Tph2*$^{+/-}$ compared to *Tph2*$^{+/+}$ mice. Full depletion of *Tph2*, was associated with 175 DEGs, with 53 genes (30.3 %) being down- and 122 genes (69.7 %) up-regulated in *Tph2*$^{-/-}$ compared to *Tph2*$^{+/+}$ mice. Over the two comparisons of gene expression in *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ with *Tph2*$^{+/+}$ mice, 12 DEGs were overlapping and showed similar significance and directionality. Furthermore, the interaction of *Tph2* deficiency and MS were interrogated. For the interaction GE1 309 DEGs were identified. Of these, 261 (84.5 %) showed a negative and 48 (15.5 %) a positive regulation. The interaction GE2 was associated

with 102 DEGs of which 76 (74.5 %) showed a negative and 26 (25.5 %) a positive regulation. 17 DEGs were overlapping over comparisons. No DEGs were overlapping between the comparison of *Tph2*$^{+/-}$ to *Tph2*$^{+/+}$ animals and their interaction with MS and 3 DEGs were overlapping between the comparison of *Tph2*$^{-/-}$ to *Tph2*$^{+/+}$ animals and their interaction with MS.

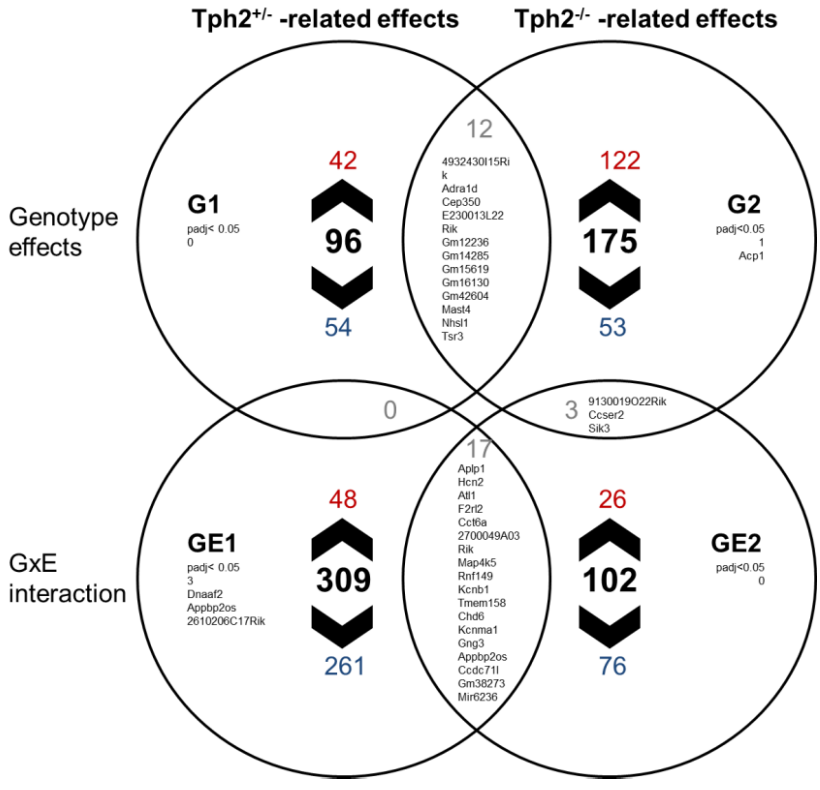

Figure 4 Differentially expressed genes, dependent on tryptophan hydroxylase 2 depletion and its interaction with maternal separation.

Comparing the effect of the tryptophan hydroxylase 2 (*Tph2*) genotypes, (G1 and G2, described in methods) independent of maternal separation (MS), showed *Tph2*$^{+/-}$ affected less genes, when compared to *Tph2*$^{+/+}$ animals, than the *Tph2*$^{-/-}$ genotype. For the interaction of the *Tph2* genotype with MS (GE1 and GE2, contrast described in methods) the effect was inverted. Data based on sequencing counts with red font indicating a positive and blue font indicating a negative directionality of the effects, N = 8. Genes, which occur in more than one comparison as well as genes, which show an p-value adjusted for multiple testing smaller than 0.05, are depicted by name.

Enriched pathways and GO-terms are depicted in S 3 Table. Amongst the top hits for an effect of TPH2 deficiency on gene expression we identified the “notch signalling” pathway (G1: z-score = 3.75, p = 0.001; G2: z-score = 4.03, p = 0.001). For the interaction GE1, for example “cytokine-related signalling” with amongst others the “IL-5 signalling pathway” (z-score = 3.19, p = 0.009) was found to be enriched. Pathways, enriched for the interaction GE2, were “circadian regulation” (z-score = 3.05, p = 0.010) and “translation factors” (z-score = 2.99, p = 0.017).

DNA methylation

DNA methylation profiles were shown to be affected by *Tph2* genotype and its interaction with MS. Some of these DMLs were associated to the same gene. The top 10 DMLs for each contrast are depicted in S4 Table. G1 was associated with 1409 DMLs. Of these 696 (49.4 %) showed decreased and 713 (50.6 %) showed increased methylation in *Tph2* $^{+/-}$ compared to *Tph2*$^{+/+}$ mice. Full depletion of TPH2, was associated with 1448 DMLs, with 831 (57.4 %) loci decreased and 617 (42.6 %) loci increased in methylation in *Tph2*$^{-/-}$ mice. Over the two comparisons of the full and partial *Tph2* knock out 146 DMLs were overlapping and showed similar significance and directionality. Furthermore, the interaction of *Tph2* deficiency and MS were interrogated. For the interaction GE1 1596 DMLs were identified. Of these, 795 (49.8 %) showed a negative and 801 (50.2 %) a positive regulation. The interaction GE2 was related to 1403 DMLs, of which 714 (51.0 %) showed a negative and 689 (49.0 %) a positive regulation. 152 DMLs were overlapping between interactions. Over the comparison of

Tph2$^{+/-}$ to *Tph2*$^{+/+}$ animals, i.e. G1 and their interaction GE1 19 DMLs were overlapping, and 10 DMLs were overlapping between the comparison G2 and the interaction GE2.

Enriched PathVisio pathways and GO-terms are depicted in S 4 Table. In brief, pathways, associated with altered DNA methylation, were partially covering functions similar to the functions already identified for gene expression for the interaction GE1, such as “IL-5 signalling” (z-score = 3.71, p = 0.002) related pathways and functions.

Related gene expression and DNA methylation

The extent to which DMLs and DEGs were related was examined (Table 3).

Table 3 Differentially methylated loci (DMLs) that are associated with differentially expressed genes (DEGs).

Chr	start_position	end_position	symbol	Ensembl ID	biotype	insideFeature	Bm	lg2FC	lfcSE	stat	pvalue	padj
A)	**Contrast (G1): [(Tph2$^{+/-}$ MS + Tph2$^{+/-}$ C)-(Tph2$^{+/+}$ MS +Tph2$^{+/+}$ C)]**											
	DML											
11	87834179	87835062	**Lpo**	ENSMUSG00000009356	protein_coding	upstream	22.41	**-0.31**	0.10	-3.12	0.002	1.00
6	113489121	113489956	**Creld1**	ENSMUSG00000030284	protein_coding	inside	27.03	**-0.27**	0.09	-2.96	0.003	1.00
2	131536681	131537247	**Gm14285**	ENSMUSG00000087524	antisense	upstream	15.32	**0.29**	0.10	2.83	0.005	1.00
	DEG											
11	87806428	87828289	**Lpo**	ENSMUSG00000009356	protein_coding		2.70	**-0.43**	0.14	-3.07	0.002	1.00
6	113483297	113493343	**Creld1**	ENSMUSG00000030284	protein_coding		5.45	**0.37**	0.14	2.59	0.010	1.00
2	131561614	131563179	**Gm14285**	ENSMUSG00000087524	antisense		14.67	**0.39**	0.14	2.80	0.005	1.00
B)	**Contrast (G2): [(Tph2$^{-/-}$ MS + Tph2$^{-/-}$ C)-(Tph2$^{+/+}$ MS +Tph2$^{+/+}$ C)]**											
	DML											
2	131595386	131596258	**Adra1d**	ENSMUSG00000027335	protein_coding	upstream	20.08	**-0.36**	0.10	-3.45	0.001	0.97
8	13456578	13457863	**Tmem255b**	ENSMUSG00000038457	protein_coding	inside	48.33	**0.25**	0.09	2.83	0.005	1.00
2	174105696	174107016	**Npepl1**	ENSMUSG00000039263	protein_coding	upstream	31.37	**0.29**	0.10	2.91	0.004	1.00
5	27020826	27021767	**Gm16057**	ENSMUSG00000081970	pseudogene	upstream	24.91	**0.28**	0.10	2.86	0.004	1.00
7	79433526	79434438	**Gm16017**	ENSMUSG00000082400	pseudogene	upstream	21.17	**-0.27**	0.10	-2.59	0.010	1.00
8	11228793	11229581	**Gm15418**	00000085573	antisense	downstream	19.99	**-0.27**	0.10	-2.59	0.010	1.00
	DEG											
2	131545850	131562283	**Adra1d**	ENSMUSG00000027335	protein_coding		6.37	**0.49**	0.14	3.41	0.001	1.00
8	13435189	13468473	**Tmem255b**	ENSMUSG00000038457	protein_coding		133.99	**0.23**	0.08	2.93	0.003	1.00
2	174110349	174123070	**Npepl1**	ENSMUSG00000039263	protein_coding		1.53	**0.38**	0.13	3.01	0.003	1.00
5	27005940	27007621	**Gm16057**	ENSMUSG00000081970	processed_pseudogene		18.34	**-0.35**	0.12	-2.89	0.004	1.00
7	79412310	79412517	**Gm16017**	ENSMUSG00000082400	processed_pseudogene		0.68	**0.28**	0.10	2.82	0.005	1.00
8	11187755	11204503	**Gm15418**	ENSMUSG00000085573	antisense		14.33	**0.35**	0.13	2.75	0.006	1.00

C)	**Contrast (GE1): [(*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)-(*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]**											
	DML											
13	36365737	36366424	**Fars2**	ENSMUSG00000021420	protein_coding	inside	15.46	**-0.74**	0.21	-3.57	0.000	0.96
13	69457460	69458618	**Nsun2**	ENSMUSG00000021595	protein_coding	upstream	37.21	**-0.52**	0.19	-2.79	0.005	1.00
9	121477914	121478574	**Cck**	ENSMUSG00000032532	protein_coding	downstream	16.13	**0.58**	0.21	2.81	0.005	1.00
1	3583857	3584649	**Xkr4**	ENSMUSG00000051951	protein_coding	inside	17.95	**0.75**	0.21	3.60	0.000	0.96
5	90554052	90554958	**5830473C10Rik**	ENSMUSG00000070690	protein_coding	upstream	24.92	**-0.69**	0.19	-3.57	0.000	0.96
2	98661728	98665716	**Gm10801**	ENSMUSG00000075015	protein_coding	includeFeature	96234.69	**-0.44**	0.12	-3.50	0.000	1.00
7	73774794	73775963	**A830073O21Rik**	ENSMUSG00000091890	protein_coding	upstream	33.84	**0.47**	0.18	2.61	0.009	1.00
10	112610921	112611527	**Gm26596**	ENSMUSG00000097185	protein_coding	upstream	15.17	**-0.55**	0.21	-2.64	0.008	1.00
	DEG											
13	36117411	36537592	**Fars2**	ENSMUSG00000021420	protein_coding		10.02	**-0.99**	0.28	-3.59	0.000	0.08
13	69533746	69635780	**Nsun2**	ENSMUSG00000021595	protein_coding		5.67	**-0.84**	0.26	-3.18	0.001	0.14
9	121489825	121495689	**Cck**	ENSMUSG00000032532	protein_coding		1.96	**-0.74**	0.27	-2.74	0.006	NA
1	3205901	3671498	**Xkr4**	ENSMUSG00000051951	protein_coding		3.54	**-0.74**	0.29	-2.59	0.010	NA
5	90561107	90597871	**5830473C10Rik**	ENSMUSG00000070690	protein_coding		0.47	**0.51**	0.17	2.94	0.003	NA
2	98662237	98664083	**Gm10801**	ENSMUSG00000075015	protein_coding		3.17	**-0.86**	0.26	-3.34	0.001	NA
7	73737929	73741024	**A830073O21Rik**	ENSMUSG00000091890	TEC		28.22	**-0.64**	0.24	-2.63	0.008	0.35
10	112928501	112931155	**Gm26596**	ENSMUSG00000097185	protein_coding		332.56	**-0.48**	0.19	-2.59	0.010	0.37
D)	**Contrast (GE2): [(*Tph*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)-(*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]**											
	DML											
5	135031000	135031630	**Stx1a**	ENSMUSG00000007207	protein_coding	inside	18.88	**-0.56**	0.21	-2.68	0.007	1.00
2	35326274	35327079	**Stom**	ENSMUSG00000026880	protein_coding	inside	21.99	**0.59**	0.20	2.95	0.003	1.00
2	4594896	4595707	**Gm13179**	ENSMUSG00000086018	antisense	upstream	16.98	**-0.56**	0.21	-2.65	0.008	1.00

	DEG										
5	135023482	135051100	**Stx1a**	ENSMUSG00000007207	protein_coding	2.28	**-0.71**	0.27	-2.59	0.010	1.00
2	35313986	35336976	**Stom**	ENSMUSG00000026880	protein_coding	0.37	**-0.47**	0.16	-2.91	0.004	1.00
2	4586024	4587287	**Gm13179**	ENSMUSG00000086018	antisense	58.62	**0.42**	0.15	2.73	0.006	1.00

List of genes that had been affected by either tryptophan hydroxylase 2 (*Tph2*) genotype (G1 and G2; reported under A and B), gene-by-environment interaction (GE1 and GE2; reported under C and D). Data based on sequencing counts of total RNA and MBD-enriched loci (group size n = 6-8). Chr=chromosome, Bm=base mean, lg2FC= log2 fold change, lfcSE=log2 fold change standard error, stat=Wald-statistic, lincRNA=long intergenic non-coding RNA, lncRNA=long non-coding RNA, padj=adjusted p-value, TEC=to be experimentally confirmed.

Amongst the genes identified in both profiles, the predicted gene A830073O21Rik, alpha-fetoprotein-related protein (*Arg*) (ρ = -0.459, p = 0.002) and cholecystokinin (*Cck*) (ρ = -0.343, p = 0.021) showed a significant, negative relation between read counts of RNA and MBD sequencing. In addition, *Cck* RNA sequencing read counts were positively related to levels of anxiety measures in the EPM (time open arm: ρ = -0.339, p = 0.020), while *Cck*-related DNA methylation showed an opposing relationship with anxiety (time open arm: ρ = 0.381, p = 0.009; closed arm: ρ = -0.304, p = 0.040). Moreover, *Cck* expression was negatively related to activity in the open field (distance moved: ρ = -0.352, p = 0.015; distance in centre: ρ = -0.294, p = 0.045) and to threat behaviour in the first RIT session (ρ = -0.321, p = 0.032). Expression and DNA methylation of *Cck* are depicted in Fig 5.

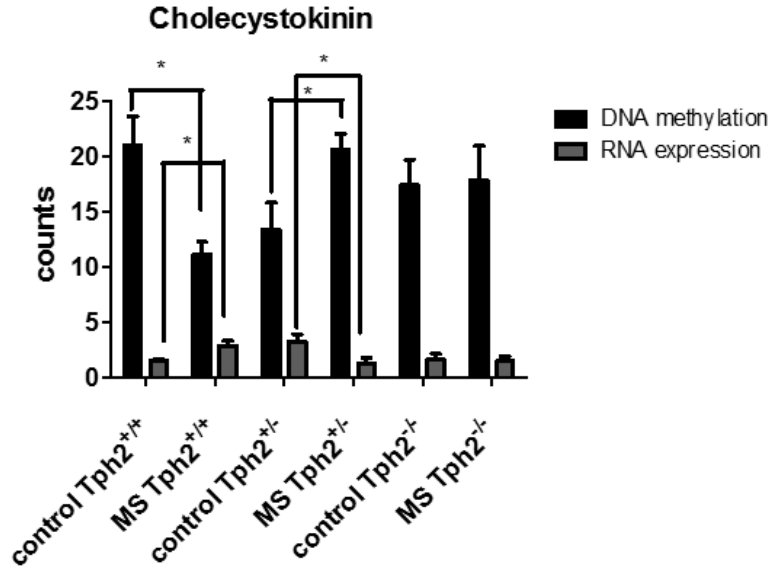

Figure 5 DNA methylation and RNA expression of cholecystokinin (*Cck*) in amygdala. Opposing effects of maternal separation (MS), dependent on the tryptophan hydroxylase 2 (*Tph2*) genotype ($p < 0.02$). Data based on sequencing counts of total RNA and MBD enriched loci (group size n = 6-8). Bars represent group means± standard errors. *p < 0.05 DNA methylation (Post hoc: Mann-Whitney U).

Discussion

In the present study, hetero- and homozygous *Tph2* knockout mice and their wildtype littermates, either left undisturbed or exposed to neonatal MS, were examined using extensive behavioural and molecular screening. While the *Tph2* genotype affected almost all investigated behaviours, physiological parameters such as FCM and the expression of numerous genes across the genome and genome-wide DNA methylation, the effects observed for MS were rather subtle.

In accordance with previously reported data (Gutknecht *et al.*, 2015), complete depletion of *Tph2* and, hence, brain 5-HT, was related to physiological alterations, such as decreased body-weight as well as altered FCM levels and stress reactivity. Firstly, we found a notable decrease in body-weight in *Tph2*$^{-/-}$ mice, which is in accordance with previous findings. Secondly, we observed a higher baseline level of FCM in *Tph2*$^{-/-}$, offspring, when compared to *Tph2*$^{+/+}$ offspring. This observation is in contrast to the previously reported findings, where Gutknecht and colleagues reported an overall lower FCM level in male Tph2$^{-/-}$ mice and might suggest a heightened activity of the HPA axis before behavioural testing in the current study. Following test exposure, *Tph2*$^{-/-}$ offspring of the current study showed a more profound change in FCM levels compared to *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ mice, with a dramatic decrease in FCM levels. Interestingly, this profile seems to correspond with the activation profile in the basolateral amygdala, described in the introduction. In their study, Waider and colleagues observed a higher

neural activation in *Tph2*$^{-/-}$ males, under home cage conditions (Waider *et al.*, 2017). This activation was abolished following exposure to a novel environment. Given the prominent role amygdala activity plays in HPA axis regulation (Herman *et al.*, 2005), these findings might hint to a potential connection between the observed FCM levels and amygdala activity.

The observed discrepancy of baseline FCM with previous findings (Gutknecht *et al.*, 2015), might be explained by various factors, such as the distinct nature of stress exposure, the genetic background of the mice (Holmes *et al.*, 2003; Mosienko, Beis, *et al.*, 2014) and the post-weaning environment (Hall, 1998). In the present study, males had been single-housed from weaning onwards. This was previously reported to alter the endocrine response in male mice (Bartolomucci *et al.*, 2003). Furthermore, post-weaning social isolation was found to alter exploratory activity (Bartolomucci *et al.*, 2003) as well as increase anxiety-related behaviours, which, only recently, was shown to be dependent on the corticotrophin releasing hormone (CRH) receptor 2 in DR (Bledsoe *et al.*, 2011). The DR CRH receptor 2, has been associated with stress-induced TPH2 activity (Donner *et al.*, 2016), suggesting a potential link with 5-HT deficiency. This might explain, why we observed an altered HPA axis activity in *Tph2*$^{-/-}$ mice but not in *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ littermates. Moreover, 5-HT synapses have been identified on CRH-releasing cells in the paraventricular nucleus (PVN) (Liposits, Phelix and Paull, 1987) and 5-HT projections innervate relevant brain structures, such as the central amygdala (Asan, Steinke and Lesch, 2013), which are mediating complex processes to the PVN (Herman and Cullinan, 1997; Van de Kar, 1997; Herman *et al.*, 2005).

In the current study, in contrast to various other reports on MS effects in rodents (Weaver *et al.*, 2004; Weinstock, 2008; Wong *et al.*, 2015), we were not able to find any effects of MS on HPA axis reactivity. One potential explanation for the lack of MS effects might be that the MS paradigm, used in this study, was insufficient, even though similar paradigms reported strong effects. A moderating factor of MS in this regard might have been the maternal response to MS exposure (Own, Iqbal and Patel, 2013). The effect of maternal behaviour and maternal stress level was associated with the phenotype, observed in adult offspring (Meaney, 2001; de Souza *et al.*, 2013; Murgatroyd *et al.*, 2015). One mediating factor of maternal behaviour is the genetic background. *Tph2*-deficient mice had previously been reported to exert altered maternal behaviour (Angoa-Pérez and Kane, 2014) and altered stress reactivity (Gutknecht *et al.*, 2015), both of which might have affected offspring development during MS.

In line with the suggestion of maternal behaviour attenuating the effects of MS, the investigated behavioural parameters showed only subtle effects for MS, while most of the reported changes were induced by *Tph2* depletion. In accordance with recently reported findings (Waider *et al.*, 2017), we found that full, but not partial, *Tph2* depletion increased general activity in a novel environment. This genotype-specific difference was exclusive to control offspring. In MS offspring the difference between genotypes did not reach significance, suggesting an increase in locomotion by MS in *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ offspring, which, in consequence, approached activity levels observed in *Tph2*$^{-/-}$ animals. Furthermore neither *Tph2* genotype (nor its interaction with MS) altered behaviour in the DLB, while in the EPM, the effects of TPH2 deficiency were very pronounced, with decreased anxiety in *Tph2*$^{-/-}$ mice, when compared to *Tph2*$^{+/+}$ mice, in particular *Tph2*$^{+/+}$ MS offspring. *Tph2*$^{+/-}$ mice showed levels of anxiety comparable to the levels observed in *Tph2*$^{+/+}$ mice. Next to the time spent on the open arms,

the overall distance covered by *Tph2*$^{-/-}$ mice on the open arms was increased when compared to both, *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ offspring. Findings in other groups, with regard to the effect of *Tph2* deficiency on anxiety, were inconsistent (reviewed in (Mosienko, Beis, *et al.*, 2014)) and most likely to be accounted for by the previously suggested factors.

Similar to anxiety-related behaviours and activity, aggression and related social behaviours were mostly independent of MS, while *Tph2* deficiency profoundly affected the performance in the RIT. *Tph2*$^{-/-}$ and *Tph2*$^{+/-}$ mice showed more offensive features such as an increased number of attacks and threat, when compared to *Tph2*$^{+/+}$ mice. Work by other groups reported amongst others a decreased attack latency and increased number of attacks in *Tph2*-deficient mice (Angoa-Pérez *et al.*, 2012; Mosienko *et al.*, 2012). Moreover, in the current study, full TPH2 deficiency was associated with deficits in social behaviours. Comparable results were reported by Kane and colleagues, who observed a decrease in several social features (Kane *et al.*, 2012). In their study, they showed that, amongst other, autism-related traits, *Tph2*$^{-/-}$ mice display less preference for socially relevant odours such as urine and, while showing a comparable habituation in a sociability test, fail dishabituation after the introduction of a novel social target. Interestingly, in the current study social behaviour was associated with FCM levels. While during the first session, home cage FCM was negatively associated with social interaction and positively associated with non-social exploration, recovery FCM was associated with social behaviour and attack latency during the fourth session. This might be indicative of an altered stress perception during the first encounter, which is in line with the observation that, while in the first session a decrease in attack latency and increase in dominance-related behaviours was observable dependent on the *Tph2* genotype, the effect did no longer reach the level of significance in the fourth session. It has been reported, that the initially observed level in aggression might not be representative of the innate aggressive potential (Winslow and Miczek, 1984; Miczek *et al.*, 2001; Caramaschi *et al.*, 2008), and the RIT has to be repeated several times, to allow the animals to reach a stable level of offensive behaviour, representing an innate state of aggressiveness. Furthermore, it is suggested that the RIT itself is perceived as stressful by the residents as they are faced with a threatening situation, leading to an increase in glucocorticoid levels (Bronson and Eleftheriou, 1965a, 1965b; Schuurman, 1980; Haller, Do and Makara, 1996; Haller *et al.*, 1998; Haller, 2014a, 2014b). As previously discussed, *Tph2* deficiency has been associated with altered HPA axis activity. Thus, despite the different nature of stress exposure in the RIT, our data might suggest that this initial increase in glucocorticoid levels might be impaired in *Tph2*$^{-/-}$ mice. *Tph2*$^{+/-}$ mice, which also showed behavioural alterations during the first RIT, when compared to *Tph2*$^{+/+}$ mice, showed no changes in FCM at baseline or following behavioural testing relative to *Tph2*$^{+/+}$ littermates. It is, thus, possible that in *Tph2*$^{-/-}$ offspring the general HPA axis activity is affected, while in *Tph2*$^{+/-}$ offspring related mechanisms might be altered in their response to aversiveness. Therefore, the observed increase in offensive behaviour might be associated with altered stress reactivity, based on two independent mechanisms for each genotype. Finally, the time, animals invested in attacking the intruder, was found to be different between groups, only during the last session and only between the control *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ offspring. None of the other groups differed in aggression. This suggests that following MS *Tph2*$^{+/+}$ offspring display an innate level of aggression comparable to *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ offspring, which is in accordance with the observations in the OF, where MS seemed to wash out the difference in activity between *Tph2*$^{+/+}$ and

Tph2$^{-/-}$ mice and supports the idea of an activity driven aggression. *Tph2*$^{+/-}$ offspring of either condition did not differ from *Tph2*$^{+/+}$ or *Tph2*$^{-/-}$ offspring.

Taken together, results of the physiological and behavioural screening suggest two major conclusions. Firstly, under aversive conditions *Tph2* deficiency altered the approach towards potentially aversive cues. In *Tph2*$^{-/-}$ animals, this could be accounted for by altered appraisal of stressful situations, which was observed for example during the EPM test, where *Tph2*$^{-/-}$ animals showed a decrease in the natural preference for the closed arms when compared to *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ littermates, independent of MS. This hypothesis found further support in the analysis of the FCM values, where *Tph2*$^{-/-}$ animals showed an initially higher FCM that dropped following behavioural testing and never came back to baseline after recovery. Most interestingly, the baseline FCM measurements correlated positively with the time spent on the open arm of the EPM, while stress and recovery FCM correlated negatively with the same measure and positively with the time spent on the closed arms. All in all, this suggests that *Tph2*$^{-/-}$ mice perceived the stressful stimulus differently when compared to their *Tph2*$^{+/+}$ counterparts and that this might be mediated via an altered regulation of the HPA axis. An alternative explanation is a dysregulation in vlPAG-DPAG signalling, caused by a lack of 5-HT, driving behaviour towards a more activity-oriented coping strategy (Paul *et al.*, 2014). Furthermore, based on the observation that the exaggerated behaviour was mostly observed in the context of aversive stimuli, a combination of altered appraisal and behavioural disinhibition might explain the observed behaviour. *Tph2*$^{+/-}$ offspring were found to display an inconclusive behavioural profile. This is most likely due to the compensatory mechanism involving decreased degradation of 5-HT (Márquez *et al.*, 2013; Mosienko, Matthes, *et al.*, 2014). *Tph2*$^{+/-}$ offspring had been observed to show reductions of only 10-20% in 5-HT levels (Mosienko *et al.*, 2012). This unproportional reduction in brain 5-HT has been suggested to be a consequence of compensatory processes, involving e.g. decreased activity of MAO-A (Mosienko, Matthes, *et al.*, 2014). Interestingly, *Mao-a* expression as well as MAO-A activity was found to be altered following early adversity, resulting in a differential susceptibility towards aversive environments (Márquez *et al.*, 2013; Wong *et al.*, 2015). Moreover, the behavioural distinction between *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ mice, or, dependent on the interrogated task, *Tph2*$^{+/+}$ mice might be dependent on the involved brain circuitries, of which each might affect unique features. Therefore, *Tph2*$^{+/-}$ offspring might show phenotypes, related to *Tph2*$^{-/-}$ offspring or*Tph2*$^{+/+}$ offspring, dependent on the task and experienced aversiveness.

Secondly, MS exerted, if at all, only subtle effects on the investigated behaviours. For example, in anxiety-related behaviours MS was found to increase the differences observed between *Tph2*$^{+/+}$ and *Tph2*$^{-/-}$ offspring, while with regard to activity, MS seemed to decrease the differences between *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ offspring. Based on current literature, we did expect an effect of MS on anxiety- and aggression-related behaviours as well as on activity (Veenema *et al.*, 2006; Veenema, Bredewold and Neumann, 2007). Furthermore, this effect of MS was hypothesised to be strain and genotype dependent. Wong and colleagues found *Tph2*-deficient offspring to be unaffected by MS, while in *Tph2*$^{+/+}$ offspring MS increased anxiety and depressive-like behaviours, with the 5-HT-deficient phenotype indicating the effects of MS (Wong *et al.*, 2015). In their study, Wong and colleagues also found altered expressions of *Mao-a* following MS in *Tph2*$^{+/+}$ offspring, supporting the hypothesis that MS exerts its effects via regulating 5-HT system functioning. Based on the previously discussed,

potential, compensatory mechanisms in *Tph2*$^{+/-}$ offspring, this suggests on the one hand altered stress susceptibility in *Tph2*$^{+/-}$ offspring (Mosienko, Matthes, *et al.*, 2014), and might, on the other hand, represent a molecular switch permissive to the consequences of MS in *Tph2*$^{+/+}$ offspring. Further studies, investigating the effects of MS on the expression of 5-HT system components found *Tph2* and *5-Htt* to be differentially regulated in the DR following early adversity (K. L. Gardner *et al.*, 2009; Katherine L. Gardner *et al.*, 2009). Moreover, MS resulted in reduced 5-HT within limbic brain regions, such as the hippocampus, in stressed offspring when compared to control offspring (Matthews *et al.*, 2001; Lee *et al.*, 2007). As a potential explanation for the lack of an observable MS effect on behaviour in the current study, we suggest a modulatory factor of early adversity, i.e. maternal behaviour (Meaney, 2001). In several studies of pre- and postnatal adversity, maternal behaviour and 5-HT functioning had been proven crucial to the behavioural outcome in affected offspring (Jones *et al.*, 2010; de Souza *et al.*, 2013; Murgatroyd *et al.*, 2015). Another factor, to be taken into account, is the inter-individual variability in animal-specific coping-style and emotional profile, which introduces a wide spectrum of behavioural responses to the experience of the same challenge (Koolhaas *et al.*, 2007).

To further investigate the underlying molecular consequences of early adversity and TPH2 deficiency, whole genome RNA expression and DNA methylation profiling were performed in the amygdala of the behaviourally screened mice. While the DNA methylation changes were relatively equal across comparisons, the number of affected DEGs differed notably, dependent on the *Tph2* genotype and MS. Comparing *Tph2*$^{-/-}$ to *Tph2*$^{+/+}$ offspring yielded about 2-fold more DEGs than when comparing *Tph2*$^{+/-}$ to *Tph2*$^{+/+}$ offspring and while in *Tph2*$^{-/-}$ offspring 70% of these DEGs displayed higher expression, an equal amount was up- and down- regulated in *Tph2*$^{+/-}$ offspring. Similarly, results regarding the contrasts GE1 and GE2, concerning the *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ genotype, respectively, suggest that *Tph2* deficiency might be altering the programming effects of MS on the methylome.

Notably, only a fraction of the differentially expressed genes were found to be associated with a differentially methylated DNA locus, and of the overlapping genes only two showed a significant, statistical correlation between expression and DNA methylation. This relatively low rate of DML-associated genes, overlapping with DEGs and the lack of significant correlation between expression and methylation of genes is most likely to be explained by the highly complex interaction of a multitude of epigenetic and structural factors such as histone modifications, DNA methylation and DNA binding factors that each are not stand-alone regulators, but rather exert their effects in concert, and create a certain chromatin state. This dynamic regulation of chromatin, in turn, changes properties of the associated transcription start sites and, consequently, gene expression (Jenuwein and Allis, 2001; Narlikar, Fan and Kingston, 2002; Barski *et al.*, 2007; Garske *et al.*, 2010; Kratz *et al.*, 2010; Fuchs *et al.*, 2011). Recent studies indicate that 1D epigenomic domains form topologically-associated 3D-domains that are acting as specialized nuclear chromatin compartments, allowing for regulatory sequences, such as enhancers, to be located far from target genes. In addition, in this way it is possible that multiple elements, which are spread out over broad regions of the genome, may orchestrate or hinder the regulation of individual genes or even gene clusters (Jost *et al.*, 2014). On the one hand, this makes it difficult to determine settings for the annotation of DMLs to genes and suggests, on the other hand, that distinct DNA methylation profiles might represent only one layer of a more complex regulation, e.g. providing a priming rather than programming and leaving the regulatory site more or

less receptive towards further regulation by, for example, histone-modifications or transcription factors (Liu *et al.*, 2016). Moreover, DNA methylation is a tissue and cell-type specific regulatory mark (Bird, 1986), rendering its analysis susceptible to altered tissue and cell-type compositions. In this study, we investigated cellular homogenate of amygdala. The amygdala comprises a complex assembly of subregions and neuronal types that are involved in the regulation of complex behaviours in a highly specific manner (Asan, Steinke and Lesch, 2013). Nevertheless, there are also exceptions and several studies investigating effects of early environment on epigenetic programming identified specific marks, regulating gene expression and, as consequence, also behaviour. One example for a direct mechanism of epigenetic programming is the epigenetic regulation of the *Nr3c1* gene, which encodes the glucocorticoid receptor (Hellstrom *et al.*, 2012).

In the current study, despite the limitations presented above, an interesting candidate-gene, *Cck*, was uncovered. The *Cck*-related DML correlated negatively with RNA expression and both *Cck* RNA expression and *Cck*-associated DNA methylation correlated most pronouncedly with behaviour in the EPM. Higher levels of anxiety were associated with a lower methylation and a higher expression of *Cck*. More specifically, *Cck* DNA methylation was found to be decreased and its expression increased by MS in *Tph2*$^{+/+}$ offspring, while in *Tph2*$^{+/-}$ offspring MS had an opposing effect, leading to an increase in DNA methylation and a decrease in expression. In *Tph2*$^{-/-}$ offspring, MS had no effect on either *Cck* DNA methylation, or expression and showed constantly low expression and high methylation levels. This coincides with the behavioural observations and might hint towards resilience to MS-induced programming in *Tph2*$^{-/-}$ offspring. CCK is a peptide that has originally been identified in the gut. It was shown to be critically involved in metabolic functions, such as the secretion of pancreatic enzymes, gut motility and gallbladder contraction. Interestingly, CCK appears in greater amounts in the brain than in the periphery and is one of the most abundant neuropeptides (Innis *et al.*, 1979; Crawley, 1985; Moran and Schwartz, 1994). *Cck*-expressing interneurons in the amygdala have been shown to be targeted by serotonergic projections (Asan, Steinke and Lesch, 2013). Furthermore, *Cck*-expressing, 5-HT1a receptor-positive interneurons in the amygdala have been associated with the display of anxiety-related behaviours (Asan, Steinke and Lesch, 2013). Moreover, it seems, that CCK is associated with specific 5-HT3 receptor-positive subpopulation of large type L interneurons in the basolateral complex of the amygdala (Mascagni and McDonald, 2007) and, furthermore, with 5-HT3 receptor-positive basket cells in the hippocampus, where they are involved in modulating anxiety following stress exposure (Gruber *et al.*, 2015). The role of CCK is exerted through its binding to two receptors, CCK1 and CCK2 (Moran *et al.*, 1986). CCK2 receptors were mainly found in the brain and shown to be involved in emotion regulation (Hughes *et al.*, 1990; Van Megen *et al.*, 1996; Wang *et al.*, 2005; Chen *et al.*, 2006). A study in rats, investigating CCK system functioning following MS, showed an increase in sensitivity towards CCK-4 injections in animals that were subjected to a separation paradigm (Greisen, Bolwig and Wörtwein, 2005). Moreover, CCK has been shown to interact with the 5-HT system. For example, in rats, administration of the selective 5-HT1a receptor antagonists (+)WAY100135 and WAY100635 resulted in an attenuation of aversion-related behaviours that were induced by CCK-8 injection (Bickerdike, Fletcher and Marsden, 1995). All in all, the involvement of *Cck* in the observed 5-HT-dependent behavioural aberrations seems to be likely and altered *Cck* regulation by MS might represent a potential mechanism of early-life adversity. Moreover, CCK has been shown to influence maternal behaviour via the CCK1 receptor (Miranda-Paiva *et al.*, 2002), which might be

involved in the observed, modest effects of MS in the current study. All dams were partially deficient for TPH2, thus, in the light of the observed results, might show increased expression of *Cck*. However, as the animals, investigated in the current study, were males, this remains to be further examined.

Next to single genes we also investigated functional patterns of differentially regulated genes. Functional annotation clustering of the top 500 hits of the differential gene expression and methylation analysis yielded associations with several pathways. Similar to the observed low overlap on candidate gene-based analyses; identified pathways were mainly unique for gene expression or DNA methylation. As previously discussed, such a low yield in overlap was not unexpected. Interestingly, however, in the interaction GE1, two of the reported pathways were identified in both analyses and were related to "cytokine signalling", namely the "interleukin 5 (IL-5) signalling" and the "chemokine signalling" pathway. 5-HT has previously been shown to be involved in regulating cytokine function in the brain (Fredericks *et al.*, 2010; Lotrich *et al.*, 2010). Furthermore, "cytokine signalling" was shown to be affected in a GxE interaction-dependent manner in mice, deficient for the 5-HTT, that were exposed to prenatal stress (van den Hove *et al.*, 2011). The reciprocal interaction between central nervous system and immune system has been subject to extensive research (Hou, Tang and Baldwin, 2013). In humans, about half of all patients undergoing long-term interferon treatment showed depressive symptoms that could be treated with selective serotonin reuptake inhibitors (Capuron *et al.*, 2002). Furthermore, the risk to develop such a clinically relevant depression following cytokine therapy was increased in people who carry the short variant of the 5-HTT gene-linked polymorphic region (Lotrich *et al.*, 2010). Thus, observations in humans and rodents suggest a strong interactive regulation of 5-HT and cytokine function. This might be affected by MS and explains why no such effects were found in *Tph2*$^{-/-}$ animals. An involvement of "cytokine signalling" in the observed heightened anxiety, in *Tph2*$^{+/+}$ following MS and in *Tph2*$^{+/-}$ offspring of either condition compared to the *Tph2*$^{-/-}$ mice might be substantial.

In the interaction GE2, related to the *Tph2*$^{-/-}$ genotype, pathways of gene expression and DNA methylation analyses did not overlap. Nevertheless, we identified one particularly interesting pathway to be enriched for gene expression terms. The pathway: "exercise induced circadian regulation" was found to be enriched, with amongst others the clock circadian regulator (*Clock*) gene, as one of its key components, being affected by the interaction. Previously *Tph2* deficiency has been associated with altered circadian rhythmic in null mutant animals (Alenina *et al.*, 2009). This observation is in line with observed alterations in activity-related behaviour and corticosterone metabolism in the present study and others (Gutknecht *et al.*, 2015). Moreover, attention deficit hyperactivity disorder (ADHD)-was found to be related to alterations in circadian rhythmic in adult humans (Van Veen *et al.*, 2010). Thus alterations in circadian regulation might link early adversity and 5-HT to the hyperactivity-related phenotype (Mogavero, Jager and Glennon, 2016).

Next to these interaction-specific pathways, "notch signalling"-associated terms were found to be altered in expression, in both *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ animals when compared to *Tph2*$^{+/+}$ animals, suggesting a rather general effect of *Tph2* deficiency through brain 5-HT depletion (Bray, 2016). Notch signalling has been associated with altered myelin structure and increased startle response (López-Juárez *et al.*, 2017), which is in line with observations in *Tph2*$^{-/-}$ animals that showed increased

reactivity to foot-shock (Waider *et al.*, 2017). Thus, altered myelination might be involved in the observed inappropriate behavioural responses.

Overall, we found that full 5-HT deficiency profoundly affected behaviour and concomitant physiology. On the one hand, brain 5-HT deficiency was associated with impaired appraisal and increased activity and, on the other hand, altered social behaviours, both in comparison to *Tph2*$^{+/+}$ offspring. However, for some of the interrogated behaviours, i.e. anxiety and activity, *Tph2*$^{+/+}$ offspring were indistinguishable from *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ offspring, following MS. The effects of MS were overall subtle and suggest stress compensation by maternal behaviour. Next to the behavioural alterations we identified, gene expression and DNA methylation profiles, associated with the *Tph2* genotype and its interaction with MS, which suggest an altered mediatory mechanism of MS, dependent on 5-HT availability in the offspring.

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Supplementary information

S 1 Table. Physiological effects of maternal separation on dams and litters.

	control	MS
Dam weight [g]		
P5	32.25 ± 0.37	32.02 ± 0.58
P10	34.81 ± 0.50	33.96 ± 0.44
P15	35.08 ± 0.38	34.94 ± 0.48
Relative pup weight [g/n]		
P5	3.24 ± 0.08	3.18 ± 0.08
P10	5.30 ± 0.10	5.26 ± 0.12
P15	7.11 ± 0.15	6.85 ± 0.12
Pup survival [%]		
P0-P15	93.24 ± 2.24	94.22 ± 1.84

Maternal separation (MS) did not affect maternal weight [g], relative pup weight [g/n] or pup survival. P= postnatal day (n = 17 per condition).

S 2 Table. Top 10 differentially expressed genes ranked by pvalue.

Gene	Ensemble ID	biotype	Chr	start	end	strand	Bm	lg2FC	lfcSE	stat	pvalue	padj
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]												
Nhsl1	ENSMUSG00000039835	protein_coding	10	18318985	18533892	+	48.47	0.39	0.10	4.09	0.0000	1.00
4930583P06Rik	ENSMUSG00000086954	lincRNA	2	124217679	124222331	-	20.18	-0.47	0.13	-3.75	0.0002	1.00
Adra1d	ENSMUSG00000027335	protein_coding	2	131545850	131562283	-	6.37	0.52	0.14	3.66	0.0002	1.00
B230110C06Rik	ENSMUSG00000097547	lincRNA	14	15437623	15452423	+	10.66	-0.48	0.13	-3.64	0.0003	1.00
Gm42604	ENSMUSG00000106526	antisense	5	96735547	96739364	-	26.63	0.37	0.10	3.59	0.0003	1.00
9130019P16Rik	ENSMUSG00000073067	processed_transcript	6	54269681	54430221	-	151.13	-0.28	0.08	-3.51	0.0004	1.00
Gm22481	ENSMUSG00000084511	misc_RNA	16	22595029	22595220	+	9.33	0.47	0.13	3.50	0.0005	1.00
Cep350	ENSMUSG00000033671	protein_coding	1	155844964	155973255	-	2.53	0.47	0.14	3.42	0.0006	1.00
D030025P21Rik	ENSMUSG00000092130	protein_coding	12	84875769	84878132	+	0.41	0.27	0.08	3.32	0.0009	1.00
Gm13361	ENSMUSG00000083777	processed_pseudogene	2	19965967	19967397	-	33.64	-0.32	0.10	-3.29	0.0010	1.00
Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]												
Acp1	**ENSMUSG00000044573**	**protein_coding**	**12**	**30893326**	**30911612**	**-**	**23.43**	**0.54**	**0.11**	**4.94**	**0.0000**	**0.02**
Cep162	ENSMUSG00000056919	protein_coding	9	87189577	87255536	-	17.79	-0.51	0.12	-4.40	0.0000	0.17
Gm35801	ENSMUSG00000104441	antisense	1	40572188	40580662	-	5.63	0.59	0.14	4.16	0.0000	0.35
Nhsl1	ENSMUSG00000039835	protein_coding	10	18318985	18533892	+	48.47	0.38	0.10	3.98	0.0001	0.45
Gm42604	ENSMUSG00000106526	antisense	5	96735547	96739364	-	26.63	0.41	0.10	3.98	0.0001	0.45
Cep350	ENSMUSG00000033671	protein_coding	1	155844964	155973255	-	2.53	0.53	0.14	3.85	0.0001	0.61
Pigm	ENSMUSG00000050229	protein_coding	1	172376546	172384099	+	0.93	0.42	0.11	3.81	0.0001	0.61
Gm16285	ENSMUSG00000090152	processed_pseudogene	12	101646186	101646532	+	1.85	0.44	0.12	3.79	0.0002	0.61
Enpp2	ENSMUSG00000022425	protein_coding	15	54838898	54920146	-	6.33	0.46	0.12	3.75	0.0002	0.62
Bysl	ENSMUSG00000023988	protein_coding	17	47599331	47611492	-	52.36	0.35	0.10	3.72	0.0002	0.65
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]												
Dnaaf2	**ENSMUSG00000020973**	**protein_coding**	**12**	**69189087**	**69198429**	**-**	**94.51**	**-0.80**	**0.18**	**-4.45**	**0.0000**	**0.04**
Appbp2os	**ENSMUSG00000085628**	**antisense**	**11**	**85234775**	**85238304**	**+**	**55.99**	**-0.94**	**0.21**	**-4.43**	**0.0000**	**0.04**

2610206C17Rik	**ENSMUSG00000085236**	**antisense**	**7**	**84689640**	**84779053**	**+**	**13.40**	**-1.12**	**0.26**	**-4.30**	**0.0000**	**0.05**
Map4k5	ENSMUSG00000034761	protein_coding	12	69803750	69893200	-	5.74	-1.14	0.28	-4.02	0.0001	0.08
Gm43137	ENSMUSG00000104970	antisense	5	110230608	110231201	-	11.58	-1.02	0.26	-3.95	0.0001	0.08
Klf3	ENSMUSG00000029178	protein_coding	5	64803388	64832901	+	3.18	-1.08	0.28	-3.91	0.0001	NA
Hcn2	ENSMUSG00000020331	protein_coding	10	79716634	79736108	+	4.52	-1.11	0.29	-3.88	0.0001	0.08
Gnb5	ENSMUSG00000032192	protein_coding	9	75311395	75344964	+	20.23	-0.88	0.23	-3.88	0.0001	0.08
Gm26798	ENSMUSG00000097578	lincRNA	15	89211559	89215012	-	81.30	-0.92	0.24	-3.87	0.0001	0.08
Gm9887	ENSMUSG00000052673	protein_coding	12	69371835	69372458	-	8.34	-0.95	0.25	-3.84	0.0001	0.08
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]												
Gm15662	**ENSMUSG00000089855**	**unprocessed_pseudogene**	**10**	**105238134**	**105238292**	**+**	**29.40**	**-1.27**	**0.25**	**-4.97**	**0.0000**	**0.02**
Gm42418	ENSMUSG00000098178	lincRNA	17	39846958	39848788	+	33395.14	-0.66	0.15	-4.35	0.0000	0.22
Gm38020	ENSMUSG00000103697	TEC	X	103576426	103581101	-	3.01	-0.96	0.28	-3.39	0.0007	1.00
Gm13268	ENSMUSG00000070271	processed_pseudogene	2	14626889	14627321	-	24.67	0.79	0.24	3.34	0.0008	1.00
Pex14	ENSMUSG00000028975	protein_coding	4	148960535	149099876	-	2.09	-0.86	0.27	-3.21	0.0013	1.00
2700049A03Rik	ENSMUSG00000034601	protein_coding	12	71136848	71243303	+	0.82	-0.68	0.21	-3.20	0.0014	1.00
Ppp1r12c	ENSMUSG00000019254	protein_coding	7	4481520	4501680	-	1.07	-0.70	0.22	-3.17	0.0015	1.00
Med13	ENSMUSG00000034297	protein_coding	11	86267033	86357602	-	8.61	-0.88	0.28	-3.17	0.0015	1.00
Cst7	ENSMUSG00000068129	protein_coding	2	150570415	150578944	+	9.83	-0.82	0.26	-3.13	0.0017	1.00
Gm26310	ENSMUSG00000088391	snoRNA	6	27668087	27668203	+	2.45	-0.86	0.27	-3.13	0.0018	1.00

List of differentially expressed genes (DEGs) that have been affected by either tryptophan hydroxylase 2 (*Tph2*) genotype (G1 and G2) or by gene-by-environment interaction (GE1 and GE2) of *Tph2* genotype and neonatal maternal separation (MS). Data based on sequencing counts of total RNA sequencing (group size = 8). Chr= Chromosome, Bm= basemean, lg2FC= log2 fold change, lfcSE= log fold change standard error, stat= wald-statistic, lincRNA=long intergenic non-coding RNA, lncRNA=long non-coding RNA, miscRNA=miscellaneous RNA, TEC=to be experimentally confirmed, miRNA=micro RNA, snoRNA=small nuclear RNA, G=genotype, GE=gene-by-environment interaction

S 3 Table. (A) Pathways and (B) gene ontology (GO)-terms comprising enriched terms of differentially expressed genes and methylated loci.
A)

Pathway	positive	measured	total	%	Z score	p-value	Genes
Pathways enriched for the top 500 hits of DESeq2 analysis for differential gene expression							
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]							
Notch Signalling Pathway	4	43	47	9.30	3.75	0.005	**APH1A;NOTCH3;CREBBP;MAML1**
Alzheimers Disease	4	73	91	5.48	2.41	0.025	**APH1A;GRIN2A;MAPT;ATF6**
Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]							
Notch Signalling Pathway	4	43	47	9.30	4.03	0.001	**APH1A;CREBBP;APH1B;DVL2**
mRNA processing	15	430	552	3.49	3.25	0.000	**EIF4A2;RBM17;RBM39;SNRPN;SSB;MYEF2;DDX3X;MAK16;GATC;CLK4;SBNO1;EXOSC5;ZMAT2;TARDBP**
IL-6 signalling Pathway	5	99	100	5.05	2.75	0.005	**MAP2K4;CREBBP;CD40;FES;JAK1**
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]							
IL-5 Signalling Pathway	4	65	70	6.15	3.19	0.004	**LYN;SHC2;SOCS1;JAK1**
Calcium Regulation in the Cardiac Cell	6	153	161	3.92	2.60	0.010	**GJD2;GNG3;KCNB1;PRKAR2A;GNB5;ADRA1A**
Chemokine signalling pathway	6	166	199	3.61	2.37	0.022	**LYN;SHC4;GNG3;SHC2;GNB5;CCR5**
EGFR1 Signalling Pathway	6	175	177	3.43	2.23	0.030	**JAK1; PITPNA; JUND1; LOC382523; MTA2; SOCS1**
Electron Transport Chain	4	97	115	4.12	2.22	0.034	**COX8A;ATPIF1;ATP5D;ATP5J**
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]							
Exercise-induced Circadian Regulation	4	47	49	8.51	3.05	0.010	**VAPA;CBX3;GFRA1;CLOCK**
Exercise-induced Circadian Regulation	4	47	50	8.51	3.05	0.010	**VAPA;CBX3;GFRA1;CLOCK**
Translation Factors	4	48	51	8.33	2.99	0.008	**EIF5A;EIF5;EEF1A2;EIF2S2**
Primary Focal Segmental Glomerulosclerosis FSGS	4	67	77	5.97	2.19	0.034	**DNM1; PLAUR; CDH2; CDKN1B**
Myometrial Relaxation and Contraction Pathways	7	151	162	4.64	2.17	0.030	**ADCY2; GNG3;IGFBP5;GRK6; LPAR1; ATF5**
Pathways enriched for the top 500 hits of DESeq2 analysis for differential MBD enrichment							
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]							
TGF Beta Signalling Pathway	4	46	53	8.70	3.72	0.003	**SMAD2;IFNG;TGFBR2**
PodNet: protein-protein interactions in the podocyte	10	271	316	3.69	2.66	0.011	**SMAD2;SCEL;MYO1E;RXRA;CDH3;SMURF1;CAPZA2;RAPGEF2;ARHGAP24;TGFBR2**

Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]							
MicroRNAs in Cardiomyocyte Hypertrophy	6	85	109	7.06	3.07	0.009	**RCAN1;PRKCB;MIR23A;LRP5;HDAC9;IKBKE**
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]							
IL-7 Signalling Pathway	4	35	45	11.43	4.18	0.001	**GSK3B;STAT1;IRF1;FOXO3**
IL-5 Signalling Pathway	5	60	70	8.33	3.71	0.001	**GSK3B;SPRED1;SYK;STAT1;FOXO3**
Focal Adhesion-PI3K-Akt-mTOR-signalling pathway	12	273	331	4.40	3.16	0.004	**CSF1R;GSK3B;CHRM1;VWF;LAMA1;SLC2A4;PIK3CB;FOXO3; COL1A1;FGF9;CREB5;ITGA9**
Wnt Signalling Pathway	4	55	62	7.27	2.97	0.007	**GSK3B;FZD7;PRKCD;PRKCQ**
IL-6 signalling Pathway	5	83	100	6.02	2.81	0.013	**TEC; GSK3B;STAT1; PRKCD; FOXO3**
Wnt Signalling Pathway and Pluripotency	5	84	98	5.95	2.78	0.008	**RACGAP1; GSK3B;FZD7;PRKCD;PRKCQ**
Focal Adhesion	8	170	186	4.71	2.77	0.007	**GSK3B; PAK3;VWF;LAMA1;PIK3CB;COL1A1;ITGA9; RAP1B**
Chemokine signalling pathway	7	148	199	4.73	2.60	0.015	**CXCR5; PIK3CB; STAT1; GSK3B; RAP1B; PRKCD; FOXO3**
IL-2 Signalling Pathway	4	65	77	6.15	2.56	0.010	**PIK3CB; SYK; FOXO3; STAT1**
XPodNet - protein-protein interactions in the podocyte expanded by STRING	20	689	837	2.90	2.15	0.026	**LAMA1; KIF26A; CDK5; VWF; PIK3CB; CAMK2B; RACGAP1; EPHA4; SKAP2; PRKCD; CSF1R; PAK3; SMAD6; PVRL1; CRTAM; PAWR; OLIG2; GSK3B; A2BP1; SLC93R2**
Insulin Signalling	6	143	161	4.20	2.08	0.029	**GSK3B;PRKCD;PRKCQ;SLC2A4;PIK3CB**
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]							
XPodNet - protein-protein interactions in the podocyte expanded by STRING	19	689	837	2.76	2.46	0.015	**CAMK2B;KL;CSF1R;HSP90AA1;SYK;VDR;CRTAM;PRKCZ;TIAM1;CL DN3;FCGRT;ITGA8;BTLA;PDE3A;SLC29A4;CDC42EP1;RARB;PLEC; CDK5RAP2**

B)

GO-term	Overlap	P-value	Adjusted P-value	Z-score	Combined Score	Genes
Biological Process enriched for top 500 hits of DESeq2 analysis for differential gene expression						
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]						
peptidyl-serine autophosphorylation	12/240	0.02	0.92	-4.00	16.05	**FGR;STK10;FLT1;MAST4;INSR;PDGFB;TYRO3;SGK3;RICTOR;EPHA1;T SSK2;STK32C**
activation of Janus kinase activity	7/83	0.00	0.92	-2.92	15.61	**FGR;FLT1;LEP;INSR;MT-RNR1;PDGFB;EPHA1**
enzyme active site formation via O4'-phospho-L-tyrosine	5/46	0.01	0.92	-2.94	15.22	**FGR;FLT1;INSR;PDGFB;EPHA1**
activation of protein kinase C activity	4/25	0.00	0.92	-2.61	14.99	**INSR;MT-RNR1;LEP;PDGFB**

positive regulation of MAPK cascade involved in cell wall organization or biogenesis	6/56	0.00	0.92	-2.49	14.76	**MYDGF;FLT1;LEP;INSR;PDGFB;PDGFA**
positive regulation of ERK5 cascade	6/58	0.00	0.92	-2.54	14.60	**MYDGF;FLT1;LEP;INSR;PDGFB;PDGFA**
positive regulation of protein kinase B signalling	6/67	0.01	0.92	-2.88	14.49	**OSBPL8;MYDGF;LEP;INSR;PDGFA;RICTOR**
Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]						
regulation of adenylate cyclase activity involved in G-protein coupled receptor signalling pathway	5/41	0.00	0.94	-2.86	16.23	**GNAT2;FPR1;ADRA1D;GNAT1;PRKACB**
peptidyl-arginine methylation	4/29	0.01	0.94	-2.75	14.24	**HSPA8;PRMT8;VCP;METTL21A**
canonical Wnt signalling pathway	7/97	0.01	0.94	-2.96	13.38	**DDX3X;UBB;CHD8;DVL2;PTEN;CTNNB1;FERMT2**
Wnt signalling pathway involved in wound healing, spreading of epidermal cells	6/73	0.01	0.94	-2.71	12.54	**DDX3X;UBB;DVL2;CTNNB1;ITGA5;FERMT2**
adenylate cyclase-activating G-protein coupled receptor signalling pathway	6/85	0.02	0.94	-3.13	12.30	**GNAT2;FSHR;FPR1;ADRA1D;GNAT1;PRKACB**
detection of calcium ion	4/36	0.01	0.94	-2.72	12.02	**RYR2;AHCYL1;SYT1;TRPV6**
histone methylation	4/42	0.02	0.94	-2.86	11.13	**HSPA8;PRMT8;VCP;METTL21A**
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]						
negative regulation of protein phosphorylation	6/76	0.01	1.00	-3.16	14.00	**LYN;SOCS1;IGFBP3;FKBP8;INHBA;INSM1**
protein insertion into ER membrane	4/35	0.01	1.00	-2.65	11.97	**GRIN3B;COG7;ATL3;ATL1**
negative regulation of I-kappaB phosphorylation	5/73	0.04	1.00	-3.10	10.31	**LYN;IGFBP3;FKBP8;OTUD7A;INSM1**
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]						
nuclear export	4/21	0.00	0.92	-2.87	18.39	**EIF5A;ANP32A;PHAX;NSRP1**
negative regulation of cellular carbohydrate metabolic process by negative regulation of transcription, DNA-templated	17/412	0.03	0.92	-4.88	16.99	**ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK**
negative regulation of mating-type specific transcription, DNA-templated	17/412	0.03	0.92	-4.88	16.98	**ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK**
nitrogen catabolite repression of transcription	17/412	0.03	0.92	-4.88	16.97	**ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK**
negative regulation of transcription by pheromones	17/412	0.03	0.92	-4.88	16.97	**ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK**
negative regulation of transcription from RNA polymerase V promoter	17/412	0.03	0.92	-4.87	16.95	**ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK**
negative regulation of antisense RNA transcription	17/412	0.03	0.92	-4.87	16.94	**ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK**

chromatin organization involved in negative regulation of transcription	17/412	0.03	0.92	-4.87	16.93	ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK
negative regulation of ncRNA transcription associated with protein coding gene TSS/TES	17/412	0.03	0.92	-4.87	16.93	ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK
negative regulation of transcription during mitotic cell cycle	17/412	0.03	0.92	-4.86	16.91	ZHX2;CDKN1B;CTBP2;ZBTB14;CBX3;ARID5A;RSF1;CTCF;PA2G4;LIMD1;PEX14;SMARCA4;TFAP4;BASP1;LANCL2;ATF5;CLOCK
Molecular function enriched for top 500 hits of DESeq2 analysis for differential gene expression						
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]						
Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]						
Hsp70 protein binding	4/25	0.00	0.43	-1.57	8.99	DNAJC10;SACS;METTL21A;PPID
potassium channel inhibitor activity	4/43	0.02	0.43	-1.91	7.28	DPP10;PDZD3;RACK1;SGK3
protein phosphatase 5 binding	4/51	0.04	0.43	-1.80	5.86	VCP;RACK1;CTNNB1;JAK1
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]						
ligand-gated cation channel activity	4/50	0.04	0.79	-2.02	6.73	CHRNA1;GRIN3B;GRIK4;SLC39A1
delayed rectifier potassium channel activity	4/54	0.05	0.79	-1.99	6.15	KCNB1;KCNMA1;KCNA7;HCN2
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]						
translation elongation factor activity	5/34	0.00	0.75	-1.89	12.33	EIF5A;EIF5;EEF1A2;EIF2S2;CPEB2
mitogen-activated protein kinase kinase kinase binding	13/304	0.04	0.75	-2.90	9.14	MAP4K2;CDKN1B;CTBP2;RPS6;ATG13;ELAVL1;DNM1;ADD2;DLG1;EEF1A2;KIF13B;GNAT1;MAP3K13
mitogen-activated protein kinase kinase binding	13/305	0.04	0.75	-2.90	9.05	CDKN1B;CTBP2;RPS6;ATG13;ELAVL1;DNM1;ADD2;DLG1;EEF1A2;KIF13B;GNAT1;MAP3K13;TRIB2
transcription corepressor activity	9/174	0.03	0.75	-2.56	8.87	ZHX2;BASP1;ARID5A;CTCF;ATF5;ZMYND11;LIMD1;PEX14;SMARCA4
translation termination factor activity	4/28	0.00	0.75	-1.63	8.68	EIF5;EEF1A2;EIF2S2;CPEB2
telomeric DNA binding	10/218	0.05	0.75	-2.72	8.26	FOXB1;TFAP4;HLF;ZBTB14;SATB2;TINF2;UPF3A;CTCF;ATF5;CLOCK
Biological Process enriched for top 500 hits of DESeq2 analysis for differential MBD enrichment						
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]						
negative regulation of transforming growth factor beta receptor signalling pathway	6/62	0.00	1.00	-2.64	14.75	SMAD2;SMURF1;HTRA4;SOX11;LDLRAD4;TGFBR2
negative regulation of transforming growth factor beta receptor signalling pathway involved in primitive streak formation	5/50	0.01	1.00	-2.52	12.54	SMAD2;SMURF1;HTRA4;LDLRAD4;TGFBR2
canonical Wnt signalling pathway involved in positive regulation of epithelial to mesenchymal transition	6/83	0.02	1.00	-2.97	12.45	SMAD2;SFRP1;CDH3;MYC;GCNT2;TGFBR2

sequestering of TGFbeta in extracellular matrix	5/54	0.01	1.00	-2.57	11.97	**SMAD2;SMURF1;HTRA4;LDLRAD4;TGFBR2**
cellular response to estradiol stimulus	4/39	0.01	1.00	-2.71	11.55	**SFRP1;MYOD1;WNT8B;SSTR2**
Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]						
equilibrioception	4/26	0.00	0.96	-2.69	15.68	**OPRK1;OPRM1;MYO7A;NEUROG1**
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]						
activation of transmembrane receptor protein tyrosine kinase activity	9/112	0.00	0.67	-3.25	20.76	**PTPRT;CSF1R;TEC;RIPK3;SYK;GRAP2;NTF3;PRKCD;PIK3CB**
regulation of platelet aggregation	3/8	0.00	0.67	-2.86	20.63	**TEC;SYK;PRKCQ**
positive regulation of transcription from RNA polymerase I promoter	19/482	0.03	0.97	-5.18	18.57	**RNF20;AFAP1L2;CASZ1;POU1F1;STAT1;FZD7;HEATR1;ESRRG;EBF3;NR1D1;FOXO3;COL1A1;MLXIPL;TBL1XR1;IRF1;MAPRE3;SKAP1;CDK5RAP2;CREB5**
ephrin receptor signalling pathway	10/151	0.00	0.69	-3.32	18.35	**PTPRT;EPHA4;CSF1R;TEC;SYK;GRAP2;NTF3;PIK3CB;EFNA5;PAK3**
sevenless signalling pathway	7/72	0.00	0.67	-2.88	18.16	**PTPRT;CSF1R;TEC;SYK;GRAP2;NTF3;PIK3CB**
positive regulation of DNA-templated transcription, initiation	19/493	0.03	0.97	-5.32	18.02	**RNF20;GSK3B;AFAP1L2;CASZ1;POU1F1;STAT1;FZD7;ESRRG;EBF3;NR1D1;FOXO3;COL1A1;MLXIPL;TBL1XR1;IRF1;MAPRE3;SKAP1;CDK5RAP2;CREB5**
Tie signalling pathway	7/72	0.00	0.67	-2.92	17.95	**PTPRT;CSF1R;TEC;SYK;GRAP2;NTF3;PIK3CB**
glial cell-derived neurotrophic factor receptor signalling pathway	7/72	0.00	0.67	-2.91	17.89	**PTPRT;CSF1R;TEC;SYK;GRAP2;NTF3;PIK3CB**
brain-derived neurotrophic factor receptor signalling pathway	7/75	0.00	0.67	-2.89	17.54	**PTPRT;CSF1R;TEC;SYK;GRAP2;NTF3;PIK3CB**
ERBB signalling pathway	7/76	0.00	0.67	-2.91	17.41	**PTPRT;CSF1R;TEC;SYK;GRAP2;NTF3;PIK3CB**
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]						
Molecular function enriched for top 500 hits of DESeq2 analysis for differential MBD enrichment						
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]						
Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]						
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C])						
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]						

S 4 Table. Top 10 differentially methylated loci ranked by pvalue.

Gene	Ensembl ID	biotype	Chr	start	end	position	Bm	lg2FC	lfcSE	stat	pvalue	padj
Contrast (G1): [(Tph2$^{+/-}$C +Tph2$^{+/-}$ MS) - (*Tph2*$^{+/+}$ C +*Tph2*$^{+/+}$ MS)]												
Iltifb	**ENSMUSG00000090461**	**protein_coding**	**10**	**118258290**	**118259228**	**downstream**	**27.27**	**0.67**	**0.10**	**6.75**	**0.000**	**0.00**
Gm9046	**ENSMUSG00000096912**	**pseudogene**	**10**	**118340784**	**118344240**	**includeFeature**	**116.79**	**0.43**	**0.07**	**6.30**	**0.000**	**0.00**
Iltifb	**ENSMUSG00000090461**	**protein_coding**	**10**	**118259603**	**118260692**	**downstream**	**33.14**	**0.60**	**0.10**	**6.00**	**0.000**	**0.00**
Il22	**ENSMUSG00000074695**	**protein_coding**	**10**	**118240183**	**118241196**	**downstream**	**28.25**	**0.61**	**0.10**	**5.93**	**0.000**	**0.00**
Il22	**ENSMUSG00000074695**	**protein_coding**	**10**	**118238775**	**118239834**	**downstream**	**35.35**	**0.50**	**0.09**	**5.52**	**0.000**	**0.00**
Iltifb	**ENSMUSG00000090461**	**protein_coding**	**10**	**118252150**	**118253773**	**downstream**	**46.37**	**0.44**	**0.08**	**5.25**	**0.000**	**0.00**
Gm9048	**ENSMUSG00000094519**	**pseudogene**	**10**	**118346710**	**118349742**	**includeFeature**	**102.62**	**0.35**	**0.07**	**4.72**	**0.000**	**0.06**
Oxgr1	ENSMUSG00000044819	protein_coding	14	119803379	119804355	downstream	31.50	0.43	0.10	4.45	0.000	0.20
Il22	ENSMUSG00000074695	protein_coding	10	118244249	118245233	downstream	37.43	0.39	0.09	4.29	0.000	0.37
Contrast (G2): [(Tph2$^{-/-}$C + Tph2$^{-/-}$ MS) - (*Tph2*$^{+/+}$ C + *Tph2*$^{+/+}$ MS)]												
Iltifb	**ENSMUSG00000090461**	**protein_coding**	**10**	**118258290**	**118259228**	**downstream**	**27.27**	**1.62**	**0.10**	**15.85**	**0.000**	**0.00**
Il22	**ENSMUSG00000074695**	**protein_coding**	**10**	**118240183**	**118241196**	**downstream**	**28.25**	**1.43**	**0.10**	**13.78**	**0.000**	**0.00**
Il22	**ENSMUSG00000074695**	**protein_coding**	**10**	**118238775**	**118239834**	**downstream**	**35.35**	**1.28**	**0.10**	**13.32**	**0.000**	**0.00**
Iltifb	**ENSMUSG00000090461**	**protein_coding**	**10**	**118259603**	**118260692**	**downstream**	**33.14**	**1.22**	**0.10**	**11.94**	**0.000**	**0.00**
Gm9046	**ENSMUSG00000096912**	**pseudogene**	**10**	**118340784**	**118344240**	**includeFeature**	**116.79**	**0.86**	**0.07**	**11.92**	**0.000**	**0.00**
Gm9048	**ENSMUSG00000094519**	**pseudogene**	**10**	**118346710**	**118349742**	**includeFeature**	**102.62**	**0.75**	**0.08**	**9.76**	**0.000**	**0.00**
Mdm1	**ENSMUSG00000020212**	**protein_coding**	**10**	**118030298**	**118033370**	**upstream**	**137.85**	**0.69**	**0.07**	**9.62**	**0.000**	**0.00**
Il22	**ENSMUSG00000074695**	**protein_coding**	**10**	**118244249**	**118245233**	**downstream**	**37.43**	**0.88**	**0.09**	**9.38**	**0.000**	**0.00**
Iltifb	**ENSMUSG00000090461**	**protein_coding**	**10**	**118252150**	**118253773**	**downstream**	**46.37**	**0.80**	**0.09**	**9.18**	**0.000**	**0.00**
Iltifb	**ENSMUSG00000090461**	**protein_coding**	**10**	**118257496**	**118258288**	**downstream**	**10.61**	**0.91**	**0.10**	**8.87**	**0.000**	**0.00**
Contrast (GE1): [(Tph2$^{+/-}$ MS - Tph2$^{+/-}$ C) - (*Tph2*$^{+/+}$ MS -*Tph2*$^{+/+}$ C)]												
Gm22031	ENSMUSG00000080595	snRNA	15	3665493	3666289	upstream	17.75	0.92	0.20	4.52	0.000	0.76
Fam105b	ENSMUSG00000046034	protein_coding	15	27610452	27611540	inside	34.94	-0.80	0.19	-4.28	0.000	0.76
Gm10398	ENSMUSG00000072686	lincRNA	14	24921617	24923276	downstream	56.52	-0.69	0.16	-4.25	0.000	0.76
Gm22958	ENSMUSG00000084461	snRNA	10	123703838	123704690	downstream	18.93	0.87	0.21	4.24	0.000	0.76

Gm4845	ENSMUSG00000095238	protein_coding	1	140823020	140824311	downstream	75.56	-0.65	0.15	-4.22	0.000	0.76
A930028C08Rik	ENSMUSG00000085819	antisense	4	149375486	149376246	downstream	18.05	0.86	0.20	4.22	0.000	0.76
Mir455	ENSMUSG00000070102	miRNA	4	63252957	63253640	upstream	15.23	0.86	0.21	4.10	0.000	0.79
Gm16250	ENSMUSG00000086097	pseudogene	13	54849987	54851150	upstream	39.57	-0.75	0.18	-4.08	0.000	0.79
Gm25875	ENSMUSG00000077511	snRNA	15	60246446	60246900	downstream	15.56	-0.84	0.21	-4.07	0.000	0.79
Gm22529	ENSMUSG00000089442	snoRNA	6	25207392	25208138	upstream	18.07	-0.83	0.20	-4.07	0.000	0.79
Contrast (GE2): [(Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C) - (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)]												
Cd6	ENSMUSG00000024670	protein_coding	19	10823564	10824488	inside	18.38	0.92	0.21	4.44	0.000	1.00
Gm6116	ENSMUSG00000072874	lincRNA	5	74881207	74883156	upstream	61.52	-0.71	0.17	-4.27	0.000	1.00
Stk32b	ENSMUSG00000029123	protein_coding	5	37682719	37683485	inside	14.76	0.88	0.21	4.18	0.000	1.00
Slc7a10	ENSMUSG00000030495	protein_coding	7	35164823	35166146	upstream	57.98	0.65	0.16	4.14	0.000	1.00
Gm17034	ENSMUSG00000090293	processed_transcript	6	82745490	82746708	upstream	28.45	0.80	0.19	4.12	0.000	1.00
Gm11500	ENSMUSG00000081363	pseudogene	11	92491733	92492175	downstream	13.19	-0.86	0.21	-4.11	0.000	1.00
Cacna2d2	ENSMUSG00000010066	protein_coding	9	107390469	107391316	upstream	20.77	0.83	0.21	4.06	0.000	1.00
Gm26970	ENSMUSG00000097947	pseudogene	5	141954464	141955306	upstream	23.23	-0.78	0.19	-4.04	0.000	1.00
Rab21	ENSMUSG00000020132	protein_coding	10	115304894	115305610	inside	14.79	0.85	0.21	4.02	0.000	1.00
Stpg2	ENSMUSG00000047940	protein_coding	3	139295675	139296714	inside	32.79	-0.73	0.19	-3.95	0.000	1.00

List of differentially methylated loci (DMLs) that have been affected by either tryptophan hydroxylase 2 (*Tph2*) genotype (G1 and G2) or by gene-by-environment interaction (GE1 and GE2) of *Tph2* genotype and neonatal maternal separation (MS). Data based on sequencing counts of MBD sequencing (group size = 6-8).Chr= Chromosome, Bm= basemean, lg2FC= log2 fold change, lfcSE= log fold change standard error, stat= wald-statistic, lincRNA=long intergenic non-coding RNA, lncRNA=long non-coding RNA, miscRNA=miscellaneous RNA, TEC=to be experimentally confirmed, miRNA=micro RNA, snoRNA= small nuclear RNA.

S 5 Table. Gene expression and methylation results for the effect of environment stratified per genotype. (A) Top 10 differentially expressed genes (DEGs) (B) Top 10 differentially methylated loci (DMLs). (C) Overlap of DEGs and DMLs. Amongst the overlapping genes only the predicted gene Gm15908 showed a statistically significant correlation (ρ = -0.336, p = 0.024). (D) Pathways and gene ontology (GO)-terms comprising enriched terms of DEGs and DMLs.

A)

Gene	Ensemble ID	biotype	Chr	start	end	strand	Bm	lg2FC	lfcSE	stat	pvalue	padj
Contrast (E1): ($Tph2^{+/+}$ MS - $Tph2^{+/+}$ C)												
Ccdc71l	ENSMUSG00000090946	protein_coding	12	32378704	32382943	+	6.44	0.84	0.20	4.19	0.0000	NA
Gm38273	ENSMUSG00000103050	TEC	2	14735546	14737927	-	111.10	-0.48	0.12	-4.05	0.0001	0.10
Appbp2os	ENSMUSG00000085628	antisense	11	85234775	85238304	+	55.99	0.60	0.15	3.95	0.0001	0.10
Map4k5	ENSMUSG00000034761	protein_coding	12	69803750	69893200	-	5.74	0.79	0.20	3.92	0.0001	NA
Rab3d	ENSMUSG00000019066	protein_coding	9	21907491	21918192	-	2.12	0.72	0.19	3.86	0.0001	NA
Mir6236	ENSMUSG00000098973	miRNA	9	110281287	110281409	+	378.95	0.77	0.20	3.83	0.0001	0.10
6430573P05Rik	ENSMUSG00000102545	lincRNA	3	79287173	79289848	+	2.51	0.64	0.17	3.79	0.0002	NA
Gm9768	ENSMUSG00000108391	TEC	4	152006759	152009619	-	6.26	0.70	0.19	3.74	0.0002	NA
Hcn2	ENSMUSG00000020331	protein_coding	10	79716634	79736108	+	4.52	0.75	0.20	3.74	0.0002	NA
Ina	ENSMUSG00000034336	protein_coding	19	47014698	47025327	+	47.01	0.65	0.18	3.71	0.0002	0.10
Contrast (E2): ($Tph2^{+/-}$ MS - $Tph2^{+/-}$ C)												
Ccdc85c	ENSMUSG00000084883	protein_coding	12	108206345	108275417	-	1.13	-0.63	0.17	-3.78	0.0002	1.00
Gm10801	ENSMUSG00000075015	protein_coding	2	98662237	98664083	+	3.17	-0.66	0.18	-3.62	0.0003	1.00
Atl1	ENSMUSG00000021066	protein_coding	12	69893105	69964085	+	9.57	-0.64	0.18	-3.45	0.0006	1.00
Gm10800	ENSMUSG00000075014	protein_coding	2	98666547	98667301	-	49.72	-0.64	0.18	-3.44	0.0006	1.00
Gm2623	ENSMUSG00000106654	processed_pseudogene	5	32838129	32838414	+	25.33	0.50	0.14	3.44	0.0006	1.00
Ubxn6	ENSMUSG00000019578	protein_coding	17	56067045	56075028	-	26.21	-0.49	0.14	-3.41	0.0006	1.00
Mrps26	ENSMUSG00000037740	protein_coding	2	130563742	130568695	+	0.92	-0.51	0.15	-3.37	0.0007	1.00
Gm10718	ENSMUSG00000095186	protein_coding	9	3023547	3025218	+	2.40	-0.56	0.17	-3.31	0.0009	1.00
Gm8107	ENSMUSG00000080710	processed_pseudogene	X	57736547	57738555	-	0.19	-0.26	0.08	-3.26	0.0011	1.00
Ptms	ENSMUSG00000030122	protein_coding	6	124913681	124920103	-	13.91	-0.64	0.20	-3.23	0.0013	1.00

Contrast (E3): ($Tph2^{-/-}$ MS - $Tph2^{-/-}$ C)												
Gm15662	ENSMUSG00000089855	unprocessed_pseudogene	10	105238134	105238292	+	29.40	-0.81	0.18	-4.49	0.0000	0.22
Gm42418	ENSMUSG00000098178	lincRNA	17	39846958	39848788	+	33395.14	-0.43	0.11	-4.00	0.0001	1.00
Fam219b	ENSMUSG00000032305	protein_coding	9	57537528	57543187	+	39.47	0.45	0.13	3.51	0.0005	1.00
Nup43	ENSMUSG00000040034	protein_coding	10	7667503	7678881	+	47.44	-0.40	0.12	-3.44	0.0006	1.00
Polr3e	ENSMUSG00000030880	protein_coding	7	120917744	120947432	+	0.67	-0.47	0.14	-3.41	0.0006	1.00
Gm29587	ENSMUSG00000100192	antisense	12	74222498	74235140	-	426.48	0.25	0.07	3.38	0.0007	1.00
Snap47	ENSMUSG00000009894	protein_coding	11	59407134	59451186	-	6.59	-0.66	0.20	-3.35	0.0008	1.00
Rab39	ENSMUSG00000055069	protein_coding	9	53684110	53706232	-	3.49	-0.66	0.20	-3.26	0.0011	1.00
Mir1901	ENSMUSG00000084565	miRNA	18	11840361	11840438	-	34.40	0.45	0.14	3.22	0.0013	1.00
Anp32a	ENSMUSG00000032249	protein_coding	9	62341293	62378812	+	19.73	-0.50	0.16	-3.15	0.0016	1.00

B)

Gene	**Ensembl ID**	**biotype**	**Chr**	**start**	**end**	**location**	**Bm**	**lg2FC**	**lfcSE**	**stat**	**pvalue**	**padj**
Contrast (E1): ($Tph2^{+/+}$ MS - $Tph2^{+/+}$ C)												
Hsp90aa1	ENSMUSG00000021270	protein_coding	12	110714304	110714938	upstream	15.74	-0.68	0.15	-4.62	0.000	0.20
Gm6327	ENSMUSG00000091416	protein_coding	16	12536635	12537551	downstream	25.30	-0.62	0.13	-4.61	0.000	0.20
Gm24212	ENSMUSG00000088883	snoRNA	8	89693684	89694763	upstream	25.71	-0.67	0.15	-4.57	0.000	0.20
Zcchc6	ENSMUSG00000035248	protein_coding	13	59926640	59927725	upstream	44.40	0.57	0.12	4.55	0.000	0.20
Vmn2r-ps87	ENSMUSG00000092195	pseudogene	7	86877869	86878956	upstream	27.39	-0.63	0.14	-4.43	0.000	0.20
Tmem229a	ENSMUSG00000048022	protein_coding	6	24956649	24957280	upstream	16.46	0.65	0.15	4.42	0.000	0.20
Gm15427	ENSMUSG00000081051	pseudogene	1	95451566	95452503	upstream	30.05	-0.66	0.15	-4.42	0.000	0.20
Adk	ENSMUSG00000039197	protein_coding	14	21106529	21107307	inside	24.03	-0.62	0.14	-4.40	0.000	0.20
Gm25831	ENSMUSG00000089549	snRNA	14	97889362	97890114	downstream	12.92	-0.65	0.15	-4.40	0.000	0.20
Plxnd1	ENSMUSG00000030123	protein_coding	6	116021799	116022648	upstream	30.41	-0.63	0.14	-4.39	0.000	0.20
Contrast (E2): ($Tph2^{+/-}$ MS - $Tph2^{+/-}$ C)												
Galnt16	ENSMUSG00000021130	protein_coding	12	80524853	80525617	inside	17.26	-0.71	0.15	-4.82	0.000	0.10
Fzd7	ENSMUSG00000041075	protein_coding	1	59486429	59487272	overlapEnd	24.91	-0.69	0.14	-4.88	0.000	0.10

Slitrk6	ENSMUSG00000045871	protein_coding	14	110984865	110985502	upstream	17.04	-0.68	0.14	-4.74	0.000	0.10
Gm24525	ENSMUSG00000064851	snRNA	5	7055141	7055703	downstream	15.64	-0.69	0.15	-4.73	0.000	0.10
Ggact	ENSMUSG00000041625	protein_coding	14	122927365	122928075	upstream	15.70	-0.67	0.15	-4.55	0.000	0.18
Spry2	ENSMUSG00000022114	protein_coding	14	105865850	105866907	downstream	28.73	-0.61	0.13	-4.51	0.000	0.18
Klhdc7a	ENSMUSG00000078234	protein_coding	4	139989955	139990786	upstream	27.13	0.62	0.14	4.50	0.000	0.18
A830009L08Rik	ENSMUSG00000097182	lincRNA	13	91377288	91377983	inside	20.85	-0.63	0.14	-4.40	0.000	0.25
Gm20386	ENSMUSG00000092603	pseudogene	6	111013145	111014219	downstream	47.52	0.57	0.13	4.37	0.000	0.26
Nmd3	ENSMUSG00000027787	protein_coding	3	69765224	69765858	downstream	14.57	-0.64	0.15	-4.34	0.000	0.27
Contrast (E3): (Tph2$^{-/-}$ MS - Tph2$^{-/-}$ C)												
Gm826	ENSMUSG00000074623	protein_coding	2	160202165	160202839	downstream	23.75	-0.62	0.14	-4.41	0.000	0.69
Gm17034	ENSMUSG00000090293	processed_transcript	6	82745490	82746708	upstream	28.45	0.60	0.14	4.31	0.000	0.69
Cntfr	ENSMUSG00000028444	protein_coding	4	41681337	41682307	inside	36.34	0.59	0.14	4.22	0.000	0.69
Gm21824	ENSMUSG00000096671	protein_coding	Y	84700338	84701415	upstream	42.58	0.56	0.13	4.21	0.000	0.69
Mroh2a	ENSMUSG00000079429	protein_coding	1	88223444	88225514	upstream	59.80	-0.61	0.14	-4.20	0.000	0.69
Cldn11	ENSMUSG00000037625	protein_coding	3	31173716	31174450	downstream	17.87	-0.61	0.15	-4.14	0.000	0.69
Gm11484	ENSMUSG00000083080	pseudogene	4	66316025	66316932	upstream	21.69	-0.61	0.15	-4.14	0.000	0.69
4930407G08Rik	ENSMUSG00000086713	lincRNA	4	107948879	107950244	upstream	44.52	0.52	0.13	4.10	0.000	0.69
Speer7-ps1	ENSMUSG00000089871	processed_transcript	5	15674064	15675263	upstream	75.22	-0.56	0.14	-4.09	0.000	0.69
Gm13576	ENSMUSG00000081065	pseudogene	2	64631963	64633375	upstream	69.51	0.51	0.13	4.07	0.000	0.69

C)

symbol	**ENSMUSG**	**biotype**	**Chr**	**Start**	**End**	**Position**	**Bm**	**lg2FC**	**lfcSE**	**stat**	**pvalue**	**padj**
Contrast (E1): (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)												
DML												
Ppp1r12c	ENSMUSG00000019254	protein_coding	7	4507489	4509334	upstream	54.82	**0.37**	0.14	2.60	0.009	0.53
Fars2	ENSMUSG00000021420	protein_coding	13	36161446	36161906	inside	14.05	**-0.43**	0.15	-2.90	0.004	0.50
Nfatc2	ENSMUSG00000027544	protein_coding	2	168553125	168555836	inside	92.38	**0.32**	0.10	3.20	0.001	0.44
Ablim3	ENSMUSG00000032735	protein_coding	18	61921725	61922847	upstream	33.11	**-0.45**	0.13	-3.43	0.001	0.39

Cpeb2	ENSMUSG00000039782	protein_coding	5	43096646	43098032	upstream	113.71	**0.45**	0.13	3.35	0.001	0.42
Hs2st1	ENSMUSG00000040151	protein_coding	3	144433048	144433849	inside	20.85	**-0.43**	0.14	-3.08	0.002	0.45
Inadl	ENSMUSG00000061859	protein_coding	4	98411976	98413042	inside	29.61	**-0.41**	0.14	-2.96	0.003	0.48
Gm16122	ENSMUSG00000085673	antisense	5	140001521	140002939	upstream	51.61	**0.42**	0.13	3.17	0.002	0.45
Gm14285	ENSMUSG00000087524	antisense	2	131558186	131560051	upstream	65.54	**0.35**	0.11	3.24	0.001	0.44
1700024N20Rik	ENSMUSG00000091154	antisense	8	27047506	27048367	upstream	17.16	**-0.48**	0.15	-3.32	0.001	0.43
A830073O21Rik	ENSMUSG00000091890	protein_coding	7	73774794	73775963	upstream	33.84	**-0.42**	0.13	-3.28	0.001	0.43
Gm21769	ENSMUSG00000094614	pseudogene	8	19968278	19969292	upstream	31.75	**0.44**	0.14	3.16	0.002	0.45
Gm26571	ENSMUSG00000096953	protein_coding	12	83967037	83967929	upstream	35.65	**0.35**	0.13	2.64	0.008	0.53
A730020E08Rik	ENSMUSG00000097924	lincRNA	6	61984389	61985607	upstream	45.07	**0.34**	0.13	2.59	0.009	0.53
DEG												
Ppp1r12c	ENSMUSG00000019254	protein_coding	7	4481520	4501680		1.07	**0.45**	0.16	2.87	0.004	NA
Fars2	ENSMUSG00000021420	protein_coding	13	36117411	36537592		10.02	**0.65**	0.19	3.36	0.001	NA
Nfatc2	ENSMUSG00000027544	protein_coding	2	168476410	168601657		1.63	**0.48**	0.18	2.70	0.007	NA
Ablim3	ENSMUSG00000032735	protein_coding	18	61799395	61911852		0.36	**0.30**	0.11	2.70	0.007	NA
Cpeb2	ENSMUSG00000039782	protein_coding	5	43233170	43289724		4.97	**0.63**	0.20	3.14	0.002	NA
Hs2st1	ENSMUSG00000040151	protein_coding	3	144429706	144570181		0.80	**0.40**	0.15	2.67	0.008	NA
Inadl	ENSMUSG00000061859	protein_coding	4	98395785	98719603		0.78	**0.37**	0.14	2.60	0.009	NA
Gm16122	ENSMUSG00000085673	antisense	5	140074105	140085512		1.48	**-0.50**	0.18	-2.85	0.004	NA
Gm14285	ENSMUSG00000087524	antisense	2	131561614	131563179		14.67	**-0.51**	0.20	-2.59	0.009	0.34
Proscos	ENSMUSG00000091154	antisense	8	27042377	27043042		82.03	**0.33**	0.12	2.68	0.007	0.29
A830073O21Rik	ENSMUSG00000091890	TEC	7	73737929	73741024		28.22	**0.47**	0.17	2.74	0.006	0.25
Gm21769	ENSMUSG00000094614	pseudogene	8	20122365	20126624		0.23	**0.27**	0.09	2.95	0.003	NA
Gm26571	ENSMUSG00000096953	protein_coding	12	83950690	83951355		16.08	**0.74**	0.20	3.70	0.000	0.10
A730020E08Rik	ENSMUSG00000097924	bidirectional_promoter_lncRNA	6	61172293	61180810		1.57	**0.57**	0.18	3.21	0.001	NA
Contrast (E2): (*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)												
DML												

Zc3h18	ENSMUSG00000017478	protein_coding	8	122383571	122384189	inside	15.13	**-0.43**	0.15	-2.91	0.004	0.87
AB041806	ENSMUSG00000046109	protein_coding	4	138387847	138388373	upstream	12.72	**0.44**	0.15	2.99	0.003	0.87
Chst8	ENSMUSG00000060402	protein_coding	7	34770073	34770981	inside	25.74	**-0.57**	0.14	-4.24	0.000	0.31
Gm10801	ENSMUSG00000075015	protein_coding	2	98661728	98665716	includeFeature	96234.69	**-0.28**	0.09	-3.17	0.002	0.87
Gm11343	ENSMUSG00000081707	pseudogene	13	24150727	24151455	upstream	21.15	**-0.41**	0.15	-2.82	0.005	0.91
Gm23291	ENSMUSG00000093352	miRNA	12	100020128	100021103	upstream	30.58	**-0.36**	0.13	-2.71	0.007	0.93
Gm21092	ENSMUSG00000095403	protein_coding	8	20526900	20527875	upstream	30.41	**-0.40**	0.14	-2.73	0.006	0.93
DEG												
Zc3h18	ENSMUSG00000017478	protein_coding	8	122376609	122417360		6.32	**-0.53**	0.20	-2.67	0.008	1.00
AB041806	ENSMUSG00000046109	antisense	4	138395198	138397714		50.23	**-0.43**	0.16	-2.68	0.007	1.00
Chst8	ENSMUSG00000060402	protein_coding	7	34674468	34812711		0.20	**-0.25**	0.09	-2.85	0.004	1.00
Gm10801	ENSMUSG00000075015	protein_coding	2	98662237	98664083		3.17	**-0.66**	0.18	-3.62	0.000	1.00
Gm11343	ENSMUSG00000081707	processed_pseudogene	13	24195897	24196495		4.70	**0.55**	0.20	2.75	0.006	1.00
Gm23291	ENSMUSG00000093352	miRNA	12	99982775	99982852		5.90	**0.55**	0.20	2.73	0.006	1.00
Gm21092	ENSMUSG00000095403	protein_coding	8	20550331	20581999		0.90	**-0.49**	0.15	-3.21	0.001	1.00
Contrast (E3): (*Tph2*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)												
DML												
Kif21a	ENSMUSG00000022629	protein_coding	15	91114778	91115845	upstream	49.38	**0.36**	0.13	2.69	0.007	0.96
Ctsf	ENSMUSG00000083282	protein_coding	19	4861846	4862757	downstream	27.95	**0.45**	0.14	3.12	0.002	0.85
Gm15908	ENSMUSG00000086786	processed_transcript	13	34667759	34668513	downstream	21.70	**-0.42**	0.14	-2.91	0.004	0.90
Gm16529	ENSMUSG00000097882	antisense	8	77448272	77449810	upstream	89.73	**0.40**	0.13	3.02	0.003	0.87
DEG												
Kif21a	ENSMUSG00000022629	protein_coding	15	90933276	91049948		6.80	**-0.57**	0.20	-2.82	0.005	1.00
Ctsf	ENSMUSG00000083282	protein_coding	19	4855129	4860912		0.37	**-0.31**	0.11	-2.72	0.007	1.00
Gm15908	ENSMUSG00000086786	processed_transcript	13	34652923	34666774		1.60	**0.49**	0.18	2.67	0.008	1.00
0610038B21Rik	ENSMUSG00000097882	antisense	8	77517056	77523898		17.98	**0.46**	0.17	2.70	0.007	1.00

D)

Pathvisio Pathway	positive	measured	total	%	Z score	p-value	Genes
Top 500 hits of DESeq2 analysis for differential gene expression							
Contrast (E1): (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)							
Calcium Regulation in the Cardiac Cell	7	153	161	4.58	2.82	0.003	**GJD2;RYR1;GNG3;PLCB3;KCNB1;GNB5;ADRA1A**
Chemokine signalling pathway	7	166	199	4.22	2.58	0.007	**GNG3;SHC2;PLCB3;STAT2;CCL5;GNB5;CCR10**
Myometrial Relaxation and Contraction Pathways	6	151	162	3.97	2.22	0.020	**GABPA; GABPB1; GNG3; GNB5; PLCB3; RYR1**
Wnt Signalling Pathway	4	57	62	7.02	3.15	0.005	**WNT10A;FZD2;WNT16;FBXW2**
Wnt Signalling Pathway and Pluripotency	4	91	98	4.40	2.03	0.029	**WNT10A;FZD2;WNT16;FBXW2**
Contrast (E2): (*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)							
Cytoplasmic Ribosomal Proteins	4	78	81	5.13	2.61	0.016	**RPL4;RPS8;RPL36A;RPL23A**
Electron Transport Chain	4	97	115	4.12	2.10	0.039	**COX8A;ATPIF1;ATP5D;ATP5L**
Contrast (E3): (*Tph2*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)							
Exercise-induced Circadian Regulation	5	47	50	10.64	4.12	0.000	**SF3A3;ZFR;GFRA1;NR1D1;CLOCK**
Exercise-induced Circadian Regulation	4	47	49	8.51	3.09	0.008	**SF3A3;ZFR;GFRA1;CLOCK**
Primary Focal Segmental Glomerulosclerosis FSGS	5	67	77	7.46	3.10	0.010	**TRPC6;COL4A4;TLN1;TLR4;DNM1**
SIDS Susceptibility Pathways	5	54	66	9.26	3.70	0.003	**MAOA;RORA;HTR2A;NFKB1;NKX2-2**
Toll Like Receptor signalling	4	33	34	12.12	4.04	0.004	**IKBKB;TICAM1;TLR4;NFKB1**
Top 500 hits of DESeq2 analysis for differential MBD enrichment							
Contrast (E1): (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)							
TYROBP Causal Network	4	51	60	7.84	3.67	0.004	**HLX; STAT5A; CREB312; MAF**
XPodNet - protein-protein interactions in the podocyte expanded by STRING	18	689	837	2.61	2.46	0.013	**KANK2;GABRB2;HSP90AA1;SMAD4;CRTAM;CRIM1;SMAD6;CMIP ;PPP3CA;TIAM1;EDNRA;WIF1;AKT3;TCF3;ATG7;SKAP2;CDK5RA P2**
Contrast (E2): (*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)							
Adipogenesis genes	6	120	133	5.00	2.49	0.021	**TLE3;E2F1;SPOCK1;TWIST1;SLC2A4;PPARA**
Non-odorant GPCRs	8	243	267	3.70	2.08	0.037	**OPRK1;ADRB1;CELSR3; CXCR5; RXFP4; FZD7; PROKR1; EDNRB**
Contrast (E3): (*Tph2*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)							

Wnt Signalling Pathway NetPath		5	89	110	5.62	2.99	0.008	**CCND1;CTBP2;MYB;CDK1;WNT7A**

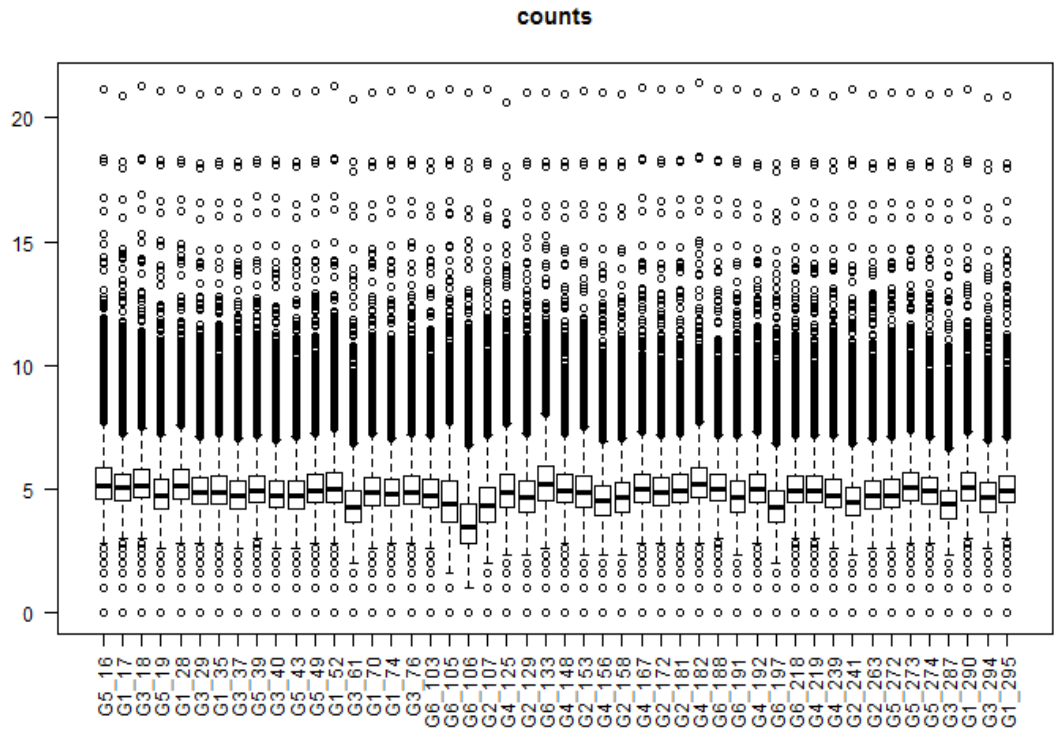
EnrichR

GO-term	Overlap	P-value	Adjusted P-value	Z-score	Combined Score	Genes
Biological Process top 500 hits of DESeq2 analysis for differential gene expression						
Contrast (E1): (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)						
protein insertion into ER membrane	4/35	0.01	0.99	-2.65	11.99	**GRIN3B;COG7;ATL3;CNTLN**
Contrast (E2): (*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)	n.s.					
Contrast (E3): (*Tph2*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)						
regulation of canonical Wnt signalling pathway involved in controlling type B pancreatic cell proliferation	4/21	0.00	0.90	-2.72	17.43	**AMER3;LRRK2;NR1D1;PSEN1**
negative regulation of neuron apoptotic process	16/399	0.04	0.90	-4.93	15.38	**EGLN2;HSPA5;LRRK2;GBA;ASNS;DHCR24;PA2G4;PSEN1;NFKB1;IKBKB;HMGN5;MT-RNR2;ERC1;ATF5;HSPA1B;AAMDC**
positive regulation of NF-kappaB transcription factor activity	10/189	0.02	0.90	-3.54	13.70	**IKBKB;PSMA6;CEBPG;ERC1;TLR4;CLOCK;HSPA1B;NFKB1;SMARCA4;NKX2-2**
Molecular function top 500 hits of DESeq2 analysis for differential gene expression						
Contrast (E1): (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)						
estrogen response element binding	10/208	0.04	0.87	-2.75	9.08	**YY1;JUND;TFAP4;H2AFY2;KMT2A;BASP1;TRIM24;CTCF;GABPB1;GABPA**
transcription regulatory region sequence-specific DNA binding	11/242	0.04	0.87	-2.74	8.73	**YY1;JUND;TFAP4;H2AFY2;KMT2A;BASP1;SOX11;CTCF;GABPB1;MTA2;GABPA**
Contrast (E2): (*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)						
ligand-gated cation channel activity	5/50	0.01	0.95	-2.03	9.80	**CHRNA1;GRIN3B;P2RX2;MCOLN1;SLC39A1**
glycine betaine-activated nonselective monovalent cation channel activity	4/47	0.03	0.95	-1.97	6.93	**CHRNA1;GRIN3B;P2RX2;MCOLN1**
Contrast (E3): (*Tph2*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)						
cadherin binding	14/275	0.01	0.55	-2.77	12.84	**MKL2;YWHAE;HSPA5;PSEN1;ATXN2L;DLG1;STK24;CAPZB;ARFIP1;FXYD5;ERC1;TLN1;ARGLU1;EPS15**
cadherin binding involved in cell-cell adhesion	14/294	0.02	0.55	-2.78	11.40	**MKL2;YWHAE;HSPA5;PSEN1;ATXN2L;DLG1;STK24;CAPZB;ARFIP1;FXYD5;ERC1;TLN1;ARGLU1;EPS15**
RNA polymerase I CORE element sequence-specific DNA binding	5/45	0.01	0.55	-1.81	9.55	**BAZ2A;RORA;NR1D1;CHD2;SMARCA4**
Rho guanyl-nucleotide exchange factor activity	5/52	0.01	0.55	-1.94	9.05	**NET1;RASGRF2;ARHGEF17;ARHGEF7;ARHGEF6**

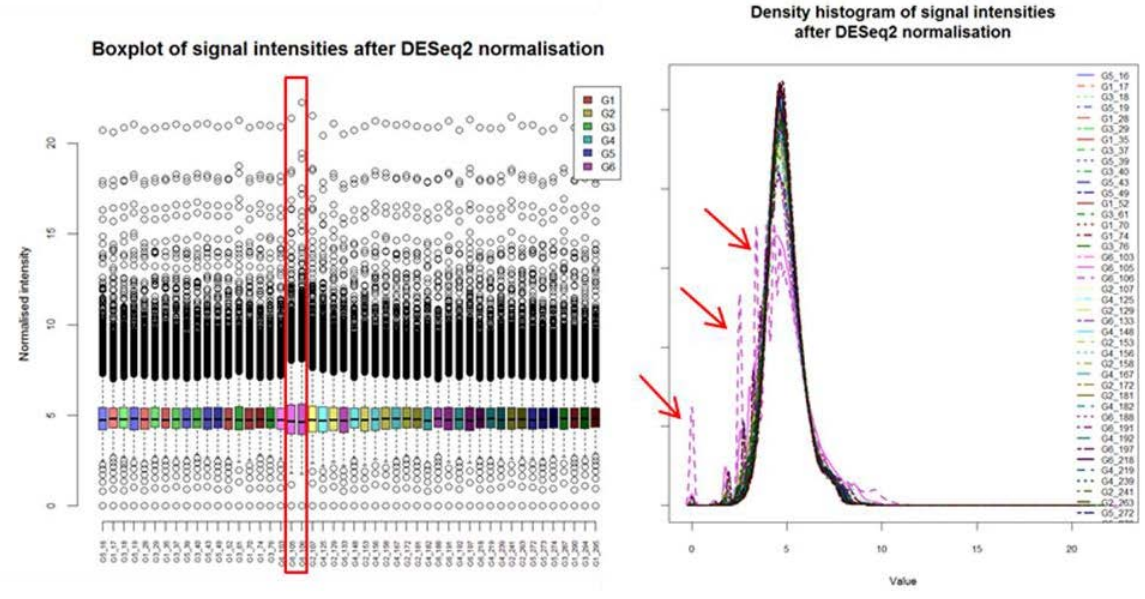
Rac guanyl-nucleotide exchange factor activity	5/56	0.01	0.55	-2.02	8.78	**NET1;RASGRF2;ARHGEF17;ARHGEF7;ARHGEF6**
K63-linked polyubiquitin modification-dependent protein binding	4/36	0.01	0.55	-1.84	8.12	**RNF169;FAAP20;EPS15;OPTN**
creatine kinase activity	4/65	0.08	0.55	-2.71	6.87	**CKMT2;PDPK1;LRRK2;CCL3**
Biological Process top 500 hits of DESeq2 analysis for differential MBD enrichment						
Contrast (E1): (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)	n.s.					
Contrast (E2): (*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)	n.s.					
Contrast (E3): (*Tph2*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)						
embryonic digit morphogenesis	4/26	0.00	0.99	-2.99	16.85	**FBXW4;CYP26B1;WNT7A;GLI3**
negative regulation of ERK1 and ERK2 cascade	5/63	0.02	0.99	-2.67	10.52	**CAV3;SYK;SPRY2;EGFR;CYR61**
Molecular function top 500 hits of DESeq2 analysis for differential MBD enrichment						
Contrast (E1): (*Tph2*$^{+/+}$ MS - *Tph2*$^{+/+}$ C)	n.s.					
Contrast (E2): (*Tph2*$^{+/-}$ MS - *Tph2*$^{+/-}$ C)						
transcriptional repressor activity, RNA polymerase II core promoter proximal region sequence-specific binding	7/116	0.02	0.75	-2.41	9.01	**SP2;MYT1L;TSHZ2;ARID5B;EN1;FOXO3;PPARA**
RNA polymerase II transcriptional repressor activity, metal ion regulated core promoter proximal region sequence-specific binding	5/77	0.04	0.75	-2.15	6.91	**SP2;MYT1L;TSHZ2;EN1;PPARA**
phosphatidylinositol-4,5-bisphosphate binding	4/53	0.04	0.75	-2.01	6.47	**GSDMD;AMER2;FZD7;KCNJ1**
Contrast (E3): (*Tph2*$^{-/-}$ MS - *Tph2*$^{-/-}$ C)	n.s.					

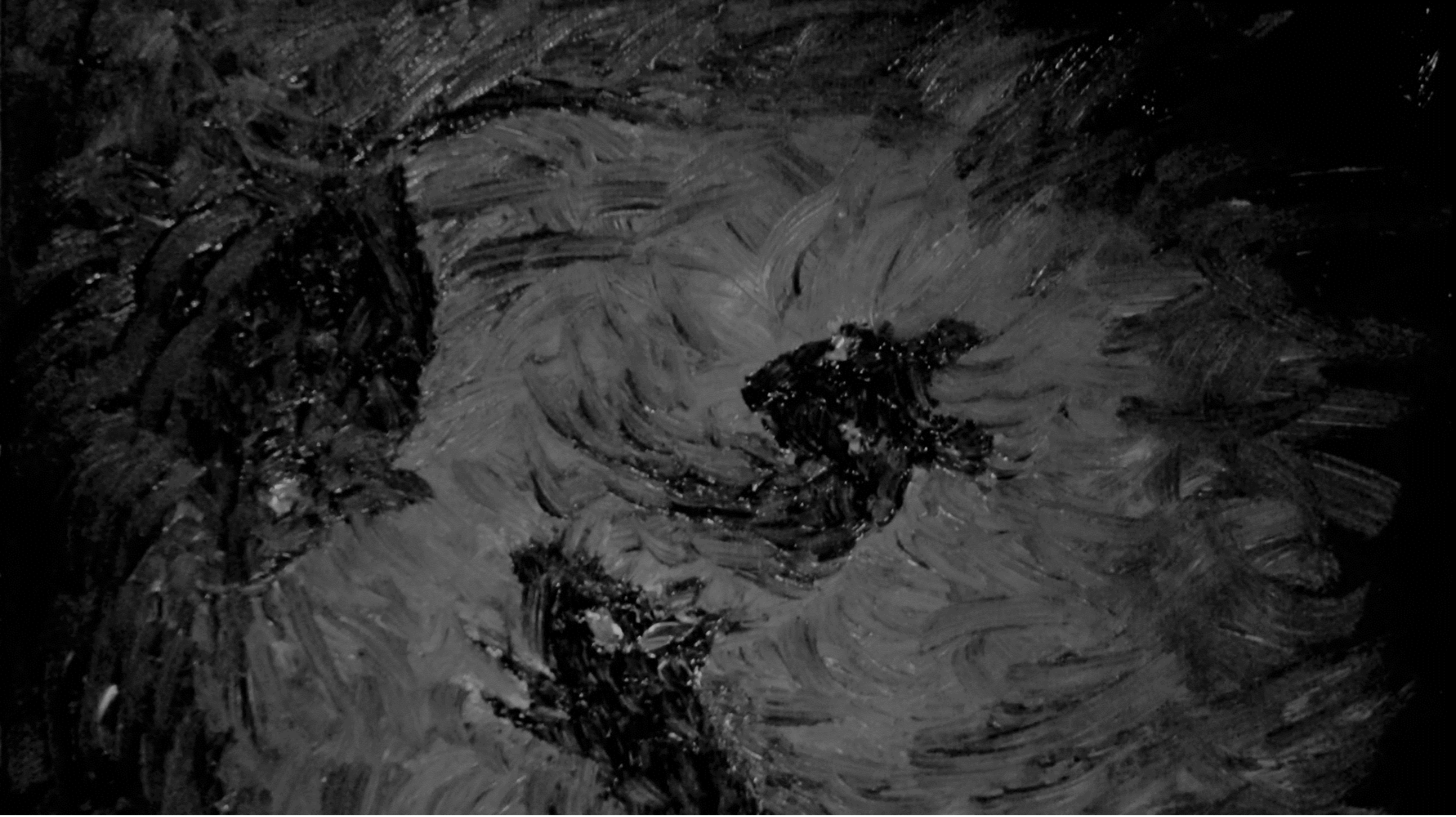
Chr= Chromosome, Bm= basemean, lg2FC= log2 fold change, lfcSE= log fold change standard error, stat= wald-statistic, lincRNA=long intergenic non-coding RNA, lncRNA=long non-coding RNA, miscRNA=miscellaneous RNA, TEC=to be experimentally confirmed, miRNA=micro RNA, snoRNA=small nuclear RNA. E=environment, DEG=differentially expressed gene, DML=differentially methylated locus

S 6 Figure. Raw counts: Sample 106 showed a pronounced deviation in terms of total MBD sequencing counts



S 7 Figure. Post normalisation density of raw counts: the sample was still observable deviating, in addition, also sample 105 showed up as clearly different from other samples





Differential susceptibility to prenatal stress exposure in serotonin transporter-deficient mice; an epigenetic perspective

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Abstract

Early-life stress, throughout critical periods of development, is strongly linked to an increased risk for the onset of mental disorders. Other important determinants that tip the scale towards health or disease, are mainly related to an individual's genetic make-up, with certain genetic variants mediating differential susceptibility towards the effects of early-life stress. The current study addressed the role of genetic variation at the serotonin (5-hydroxytryptamine, 5-HT) transporter (5-HTT, SERT, Slc6a4) in the context of prenatal stress (PS) exposure. Dependent on the *5-Htt* genotype, offspring of PS dams showed distinct reactivity to anxiety-related tests, such as the elevated plus maze (EPM) and the 3-chamber sociability test (3-CST). Assessing an increased number of offspring, to account for the variability in the response to PS, allowed us to discriminate between socially anxious (susceptible) and socially normal, (resilient) PS offspring. To determine the molecular make-up in the hippocampus, associated with differential susceptibility to PS, hippocampal transcriptomic and genome-wide histone methylation (H3K4me3) profiling was performed. In line with the behaviourally determined differential susceptibility, transcriptome-wide hippocampal gene expression showed a distinct profile for either degree of susceptibility and was found to be dependent on the *5-Htt* genotype, with up to 30 times more genes regulated in *5-Htt*$^{+/-}$ offspring, when compared to *5-Htt*$^{+/+}$ offspring. With regard to the observed effects of differential susceptibility on the H3K4me3 a similar, in contrast, a modest effect, of the *5-Htt* genotype was observed, with about three-fold more enriched fragments in *5-Htt*$^{+/+}$ offspring when compared to *5-Htt*$^{+/-}$ offspring. All in all, the observed, differential susceptibility was distinguished by *5-Htt* genotype-dependent, specific transcriptomic and epigenetic signatures, supporting the notion of 5-HT-dependent developmental programming. The observed molecular changes hint towards the involvement of genes, in particular, related to oligodendrocyte differentiation and myelination.

Introduction

Harmful events during development, such as stress exposure *in utero*, early neglect, or childhood abuse, were identified as important contributors to an increased susceptibility to mental illness later in life. Potential determinants of altered health are, for example, maternal mental problems or perceived anxiety during pregnancy, which were found to result in altered offspring susceptibilities to the environment (Buss *et al.*, 2007; Pickles *et al.*, 2017) and changed probabilities for developing adverse traits or mental disorders in offspring, in later life (Glasheen *et al.*, 2013; Betts *et al.*, 2015). Similar observations were made with regard to acute stressful events that pregnant mothers experience during pregnancy (Li *et al.*, 2010; Class *et al.*, 2014), although the effects of maternal cortisol, measured during pregnancy, on offspring behaviour and their relations to the experienced maternal stress were inconclusive (reviewed in (van den Bergh *et al.*, 2017)). Next to these external effectors, genetic predispositions, in particular, regarding the serotonin (5-hydroxytryptamine, 5-HT) system, have been linked to altered, mental health and susceptibility to adverse factors throughout life. For example, a variation of the serotonin transporter (*5-HTT*) gene-linked polymorphic region (5-HTTLPR), which garnered much attention due to its relative abundance within the general population, was found to play a modulatory role regarding environmental effects (Lesch, 2004). Next to the modulatory role of 5-HT-related genetic variation, the 5-HT system functioning itself was shown to be altered by experiences throughout early development (Katherine L. Gardner et al. 2009a; K. L. Gardner et al. 2009b), creating a 5-HT-dependent modulatory interface that can alter the outcome of environmental stimulation. For example, 5-HT signalling was shown to be involved in relevant epigenetic processes of early experiences by facilitating the recruitment of chromatin remodelling factors and transcription factors, such as cAMP response element binding protein binding protein (CBP) and early growth response protein 1 (Egr1) (Hellstrom *et al.*, 2012). Consequently, an interaction of early-life stress and functioning of the 5-HT system is suggested to exert an effect on the epigenetic landscape and, through such, on gene expression and behaviour.

In a previous mouse study, we looked into the molecular mechanisms involved in 5-HTT-dependent mediation of prenatal stress (PS) exposure. Using a maternal restraint paradigm (Behan *et al.*, 2011), applied in *5-Htt*$^{+/-}$ mice (Bengel, Murphy and Andrews, 1998), we were able to show that the long-term behavioural effects of PS were partly dependent on the *5-Htt* genotype (van den Hove *et al.*, 2011). More specifically, PS offspring showed signs of increased depressive-like behaviour, when compared to non-stressed offspring, an effect that was particularly pronounced in female *5-Htt*$^{+/-}$ mice. However, even in those female 5-HTT-deficient mice, we observed a considerable degree of variation in stress coping, indicating differential susceptibility to PS exposure (Jakob *et al.*, 2014). Transcriptomic and methylomic profiling, using hippocampal tissue of the female offspring, revealed a multitude of new candidate genes related to PS and potentially mediating susceptibility or resilience to PS (van den Hove *et al.*, 2011; Jakob *et al.*, 2014). Furthermore, 5-HTT deficiency, PS and their interaction differentially affected DNA methylation at numerous gene promoters, a subset of which was concomitant with differential mRNA expression of the corresponding genes (Schraut *et al.*, 2014).

The current study aimed at further elucidating the molecular mechanisms underlying differential susceptibility to the effects of PS on a set of mood- and anxiety-related tasks. To meet this end, we employed a similar, experimental design as in previous studies, albeit by assessing an increased

number of offspring, to account for the variability in the response to PS, allowing us to discriminate between socially anxious (susceptible) and socially normal (resilient) PS offspring, and to consecutively assess the molecular make-up in the hippocampus, associated with the observed differential susceptibility to PS. In addition to a thorough behavioural screening, we employed a hippocampal transcriptomic and genome-wide H3K4me3 profiling. The H3K4me3 histone mark represents a permissive epigenetic mark (Barski *et al.*, 2007) that was previously shown to be associated with developmental programming (e.g. (Zhang *et al.*, 2013)).

Material and methods

Animals and procedures

The study was approved by the District government of Unterfranken, (Würzburg, Germany; permit number: 55.2-2531.01-93/12), and all efforts were made to minimize animal numbers and suffering. All experiments were performed in accordance with the European Parliament and Council Directive (2010/63/EU).

Animals and prenatal stress

Male *5-Htt*$^{+/-}$ mice (B6.129(Cg)-Slc6a4tm1Kpl/J; ZEMM, breeding facility, Würzburg) were put together with two C57BL6/J female mice (Charles River, Sulzfeld, Germany) each. Males were housed under 14 h/10 h light-dark cycle with lights on at 7AM - 9PM, at 21±1°C, with humidity of 45-55%. Standard rodent chow and water were available *ad libitum*. Females were allowed to adapt to the housing conditions for at least 2 weeks upon arrival. From the moment, the breeding pairs were put together, females were tested for vaginal plugs daily. Following a positive plug control, referred to as embryonic day 0 (E0), females were housed individually. Body-weight of pregnant females was determined at E0, E13 and E17 (S 7).

From E13 to E17 a subset of the pregnant females was subjected to restraint stress for 45 min, three times per day during the light phase with an interval of 2-4 h. The stress paradigm consisted of restriction in a 25 cm-high, 250 ml glass cylinder filled up to a height of 5 mm with water (hand-hot) whilst exposing them to bright light as described by Behan et al. (2011). Control animals were left undisturbed in their home cages. Following the last stress session and concomitant weighing of the dams on E17, up to postnatal day 5 (P5), dams and their respective litters were left undisturbed. Pups were counted and litters were weighed at P5. Pups were additionally weighed on P12 and P21 before weaning and on P35 and P60 after weaning. Around P25 pups were weaned and group-housed according to their genotype and condition. Female pups that were subsequently tested behaviourally, were housed in groups of 3±1 under an inverted 12 h/12 h light-dark cycle (lights on from 7PM) from P28±1 onwards. Animals were allowed to grow up undisturbed except for weekly cage changes. At approximately 9 weeks of age, behavioural testing started. Following behavioural testing and the assessment of hypothalamic-pituitary-adrenal (HPA) axis reactivity, animals were sacrificed using isoflurane followed by decapitation. Brains were harvested and immediately, carefully frozen in isopentane at -80°C.

Behavioural testing

Behavioural testing and analysis were performed by Karla-Gerlinde Schraut (PhD) and Nicole Leibold (PhD). All behavioural tests were performed in the dark-phase between 9AM and 7PM. Animals from litters that were smaller than 5 pups were excluded from testing due to the effects of litter size on development (Tanaka, 1998). Behavioural tests were performed, starting with the elevated plus maze (EPM), followed by the Porsolt swim test (PST) and sucrose preference test, and at last the 3-chamber sociability test (3-CST). For all tests, unless stated otherwise, mice were tracked using infrared light from below the respective apparatus. Trials were recorded from above, using an infrared-sensitive camera. Behavioural analysis was performed using VideoMot2 tracking software (TSE Systems, Bad Homburg, Germany). In-between trials the respective apparatus was cleaned with Terralin liquid (Schülke, Norderstedt, Germany). Overall, 20 control animals of each genotype and 36 *5-Htt*$^{+/+}$ as well as *42 5-Htt*$^{+/-}$ PS offspring were tested.

Elevated plus maze

The EPM test is a conflict anxiety test, investigating avoidance behaviours to the aversive stimulus of height. The EPM apparatus is a plus-shaped acrylic glass construct (TSE Systems, Inc., Bad Homburg, Germany) made from black opaque PERSPEX XT, semi-permeable to infrared light, with two opposing closed arms (30 cm × 5 cm) surrounded by 15 cm high walls and two opposing open arms without walls (30 cm × 5 cm, with 0.5 cm wide boundaries elevated 0.2 cm). The four arms meet in the centre to form a square of 5 cm × 5 cm. The maze was raised 62.5 cm above the ground. The test was undertaken under low light conditions. Each animal was placed in the centre facing an open arm and allowed to explore the maze for 5 min. Subsequently, time spent and distance moved in the open arms, the closed arms and the centre, as well as the number of entries into the different arms were analysed (Lister, 1987; Post *et al.*, 2011).

Porsolt swim test

The PST is associated with behavioural despair and learned helplessness in rodents (Porsolt, Le Pichon and Jalfre, 1977). Mice were placed in a 40 cm tall acrylic glass cylinder of 19 cm diameter filled up to 15 cm with warm water (31±1°C) for 10 min. Mice from one cage were tested in parallel, in visually separated cylinders. The setup was illuminated from below with a light-box. Between trials, the water was renewed and the cylinders were cleaned with Terralin. Subsequent video analysis was performed using Ethovision Pro software (Noldus, Wageningen, The Netherlands), using distance moved as indicative parameters of mobility in the PST (Behan *et al.*, 2011).

3-chamber sociability test

The 3-CST was used to examine social preference and anxiety (File, 1980). The setup consisted of an acrylic glass structure comprising 3 separate chambers, i.e. one middle chamber with two adjacent chambers of equal size, to be reached through a passage. Both side chambers contained a wire cage. Prior to testing, animals were allowed 5 min of habituation in the apparatus. Following these 5 min, a conspecific was placed into one of the small wire cages and the subject was allowed 10 min of exploration. Subsequently, the time spent in the respective chambers was manually assessed.

Sucrose preference test

Mice were single-housed for 12 h during the dark phase, from 8AM to 8PM, and presented two similar bottles, one filled with regular tap water and the other with 1% sucrose solution. To exclude the influence of a side preference, the sugar bottle was positioned on the right or left side in an alternating fashion. The bottles were prepared one day in advance to ensure that the solutions were at room temperature (RT) and to avoid the use of leaking bottles. All bottles were weighed both before and after the test. Sucrose preference was calculated as the percentage of consumed sucrose solution in view of the total volume consumed (Strekalova *et al.*, 2004).

Corticosterone response

To assess acute corticosterone response, blood was collected from the saphenous vein before (basal) and immediately after 20 min of restraint stress (stress) one week after the last behavioural test. The stress procedure was performed as described for PS. Blood samples were subsequently centrifuged and the plasma stored at -80°C. Plasma corticosterone concentrations were determined using a radioactive immunoassay (RIA) as described in more detail previously (Van den Hove *et al.*, 2006).

Extraction and sequencing of nucleic acids

For the sequencing analyses, both DNA and RNA were extracted from hippocampal tissue. Frozen brains were semi-thawed on a -6°C cooling plate and the hippocampus was rapidly dissected using a stereo microscope (Olympus Europa, Hamburg, Germany). Before extracting nucleic acids, the tissue of the left and right hippocampus was merged, powderised at -80°C and split into two homogenous portions to enable investigating RNA expression and epigenetic modifications on the DNA in the same animals.

mRNA sequencing

RNA extraction was performed using a combination of the classic phenol-chloroform method and a column-based protocol, using the commercial miRNeasy Mini kit (Qiagen, Hilden, Germany) for fatty tissue. In brief, powderised hippocampal samples were homogenized with one ice cold stainless steel bead (Qiagen, Hilden, Germany) in QIAzol lysis reagent using the TissueLyzer (Qiagen, Hilden, Germany) at 20 Hz, 60 s, 4°C. Subsequently, the homogenates were incubated at RT for 5 min, mixed with chloroform (Carl Roth, Karlsruhe, Germany), incubated for another 10 min on ice and transferred to 1.5 ml MaXtract High Density tubes (Qiagen, Hilden, Germany) for phase separation. Following phase separation by centrifugation, the aqueous phase was transferred to a new tube, mixed with 1.5 volumes ethanol (95-100%), transferred to a RNeasyMini column and washed consecutively, using the kit-specific RWT and RPE buffers following manufacturer's instructions. To minimise the risk for genomic DNA contamination, a DNase digestion step using RNase-free DNase (Qiagen, Hilden, Germany) was included. Finally, RNA was eluted with RNase free H_2O provided with the kit and stored at -80°C. RNA quality was assessed on a 1.5% agarose gel with ethidium bromide and verified using the Experion (Biorad, München, Germany) according to the manufacturer's instructions. RNA concentrations were determined using a Nanodrop (Thermo Scientific, Wilmington, Delaware, USA). RNA sequencing and all related processes were performed by IGA Technologies (Udine, Italy). Only RNA samples with a RNA quality indicator (RQI) value of 8 or higher were used for mRNA sequencing.

For the library preparation poly-A-containing mRNA was isolated from total RNA using poly-T oligo-attached magnetic beads. Subsequently, mRNA was fragmented and transcribed into cDNA. After blunting the ends, a poly-A overhang was added. Finally, adapters, containing a unique index sequence for each sample, were ligated to the cDNA fragments to allow the identification of every read. The ligation product was purified and amplified by PCR, using primers, targeting the adapters, and mRNA sequencing was performed on an Illumina HiSeq2000 sequencer (Illumina, San Diego, California, USA) with a read-depth of 30 million reads per sample and a read-length of 50 bp. The minimum, uniquely-aligned number of reads per sample analysed was 26 million. Data demultiplexing, clean up and quality control were performed by IGA Technologies. Subsequent mapping was performed by the Interdisciplinary Centre for Clinical Research (IZKF) at the University of Würzburg. Reads were mapped to the *mus musculus* GRCm38.p5 genome using STAR (Dobin *et al.*, 2013). Following mapping, the reads per position were determined using HTSeq (Anders, Pyl and Huber, 2015).

H3K4me3 chromatin-immunoprecipitation sequencing

Powderised hippocampal tissue was mildly fixed using 1% formaldehyde. The fixation was stopped after 10 min by incubating the samples with 0.125 M glycine for 10 min at RT. All following steps were performed in solutions containing protease inhibitors (Roche, Basel, Switzerland). Following quenching, the samples were washed, subsequently grinded in SDS-lysis buffer (1%SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)) and incubated on ice for 10 min. Sonication was performed at 4°C in ice water using the Biorupter UCD-200 (Diagenode, Liège, Belgium), at the low intensity setting for 2x 10 min with the setting of pulse interval at 0.5. To test shearing success and to determine DNA concentration DNA was extracted from a portion of each sample as described below. Following DNA extraction, fragment size was determined using the Bioanalyzer 2100 (Agilent, Santa Clara, California, USA) and the DNA concentration was measured using the Qubit dsDNA HS Assay kit. In case of successful shearing, 1000 ng/antibody was transferred to a fresh 2 ml tube, filled with chromatin-immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1x protease inhibitor cocktail) up to the volume of 1 ml, per sample. Subsequently, samples were precleared using herring-sperm-DNA-blocked protein A agarose beads for 30 min at 4°C on a rotor. Next, the samples were incubated with the respective primary antibody (H3K4me3: 39159, Active motif, Carlsbad, CA, USA; rabbit IgG: X090302, Vector Laboratories, Burlingame, CA, USA) overnight at 4°C on a rotor. After incubation, antibodies were captured by using herring-sperm-DNA-blocked protein A agarose beads for 2 h at 4°C on a rotor. Following antibody capture, the beads were washed for several times, with the following buffers in the listed order: Low Salt Buffer (0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt Buffer (0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl Buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris, pH 8.1) and two times with 1x TE buffer, after which the antibodies with the captured DNA were eluted from the beads, using elution buffer (0,1 M $NaHCO_3$, 1% SDS). Subsequently, the samples were de-crosslinked, using 0.2 M NaCl at 65°C overnight. Genomic DNA was isolated, using phenol/chloroform/isoamyl alcohol extraction. For this, proteinase K (Applichem, Darmstadt, Germany) was added and the samples were incubated at 55°C for 3 h. Following proteinase K treatment, samples

were incubated for 1 h at 37°C with RNAse A (Roche, Basel, Switzerland) and then mixed with 2 volumes phenol/chloroform/isoamyl alcohol solution (25:24:1). Phases were separated by centrifugation using MaXtract high-density tubes (Qiagen, Hilden, Germany). The aqueous phase was then mixed with 2 volumes phenol/isoamyl alcohol (24:1) and phases were separated as described above. Finally, DNA was precipitated with 0.1 volumes sodium acetate, 2 volumes ice cold ethanol (95-100%) and 5 µg glycogen at -20°C. In order to remove salt residues, the DNA pellets were washed with ice cold 80% ethanol using the same conditions as for the precipitation. Finally, the pellet was air-dried at RT and resuspended in 1x TE buffer. Immunoprecipitated (IP) DNA was stored at -80°C for further use and a fraction was used for quantification using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA USA) with the Qubit high sensitivity kit for double-stranded DNA (Thermo Fisher Scientific, Waltham, MA USA). Furthermore, specificity of the IP was investigated using qPCR, with Afamin (*Afm*) as negative control and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as positive control (S 5). Both primer pairs are located within the promoter region of the respective gene (see S 1-3).

The remainder of the IP DNA (see S 4) was sent to Nxt-Dx (Ghent, Belgium), who performed the sequencing. Library preparation was performed using the NEBNext Ultra II DNA Library prep kit for Illumina (NEB, Ipswitch, Massatchusettes, USA). Subsequently, the whole IP material was subjected to ends prep and ligation of Illumina adaptors. A clean up of the adaptor-ligated DNA with AMPure XP beads (Beckman Coulter) was performed without size selection. The eluted material was subjected to enrichment PCR (14 cycles) with the NEBNext Index primers, followed by clean up with AMPure XP beads. The quality of the final libraries was checked on a Bioanalyzer 2100 DNA 1000 chip (Agilent, Santa Clara, CA, USA). The concentration was determined by performing qPCR on the samples using a dilution of PhiX index3 as standard. The concentration of all indexed samples was adjusted to 10 nM and samples were pooled for sequencing. Sequencing was performed on an Illumina HiSeq4000. All samples were processed at a read-length of 50 bp with 25-30 million reads/sample, paired-end sequencing. Follow-up data demultiplexing, clean up and quality control were performed by Nxt-dx. Subsequently, reads were mapped to the *mus musculus* GRCm38.p5 genome using Bowtie2 (v2.1.0) software in end-to-end & sensitive-mode. Coverage peaks were generated using MACS 14 peak caller (Zhang *et al.*, 2008). Following peak calling, peaks were aligned and sequencing reads within overlapping peak sets were counted using the DiffBind R-package v2.0.9 (Stark and Brown, 2013). This resulted in count-tables of the enriched H3K4me3 loci. Genes were annotated based on the first nearest feature, using ChIPpeakAnno (3.8.9) (Zhu *et al.*, 2010) with TxDb.Mmusculus.UCSC.mm10.ensGene: Annotation package for TxDb object(s), R package (version 3.4.0.; 2016) and EnsDb.Mmusculus.v75 Ensembl based annotation package, R package (version 2.1.0; Maintainer: Johannes Rainer 2016). QC results are summarised in S 6. It has to be mentioned that mapping efficiency was lower than expected for some of the samples and, while no technical issues or group bias could be detected, results should be interpreted with this in mind.

Statistical analysis

For statistical analysis of behaviour and physiological measures, SPSS Statistics (IBM Deutschland GmbH, Ehningen, Germany) was used. Data were examined for normal distribution and outliers using Shapiro-Wilk test and boxplots. As several investigated parameters did not fulfil the assumptions for ANOVA, a non-parametric Kruskal-Wallis test was performed to test for main effects or interactions. Correlations were calculated using Spearman's correlation coefficient (ρ). P-values < 0.05 were considered significant. Following this first analysis, behaviours that showed a clear PS effect or interaction were investigated with a focus on inter-individual variability. In the 3-CST test this allowed for group segregation. A cut-off at 200 s (total test duration/3 [equally sized chambers]; i.e. chance level) of time spent in the target chamber was used for further analysis, to determine a group of socially normal (resilient) and socially anxious (susceptible) PS animals. Of note, the great majority of control animals spent more than 200 s in the (social) target chamber, indicative of intact sociability. For all following analyses groups were determined as follows: control, susceptible and resilient, for either *5-Htt* genotype, dependent on the performance in the 3-CST. Group comparisons were always against the corresponding control group. The effects of stress on plasma corticosterone were investigated, using repeated measures ANOVA (baseline and stress, with the 2 fixed factors genotype and resilience). To this end corticosterone data had to be log 10 transformed.

Statistical analyses of the sequencing experiments were performed using R (version 3.3.2) and RStudio ((version 1.1.383):R Core Team (2016).(https://www.r-project.org) and RStudio Team (2017).(http://www.rstudio.com)). Both differential RNA expression and H3K4me3 based enrichment in hippocampal homogenate were determined with the DESeq2 R-package (Love, Huber and Anders, 2014). For the RNA sequencing a total of 48 animals (n = 8/group) and for the H3K4me3 peak analysis 6 samples per group, a total of 36 samples was analysed. Comparisons were made, dependent on the differential susceptibility, in the context of *5-Htt* genotype. In brief, effect directions of susceptibility (SUS–control) and resilience (RES-control) were calculated for each *5-Htt* genotype independently. For the RNA sequencing experiment this resulted in lists of differentially expressed genes (DEGs), comprising the base mean of all counts per gene, the ratio on a log2 scale (log2 fold Change; lg2FC), the log fold change standard error, the Wald statistic, p-value and adjusted p-value. A similar output was generated for the determination of H3K4me3 differentially enriched loci (DELs), comprising, in addition to the fragment peaks, a list of associated genes and their features. Genes were determined differentially expressed or modified, if they showed a nominal p-value < 0.01 and lg2FC > |0.2|. In addition to focussing on RNA expression and H3K4me3 enrichment separately, the overlap of the DEGs and DEL-associated genes per comparison was investigated and a non-parametrical linear regression analysis was performed on the raw counts of overlapping RNA and ChIP sequencing genes, as well as behaviour and corticosterone levels.

Pathway enrichment analysis was conducted using the pathway analysis tool PathVisio (van Iersel *et al.*, 2008; Kutmon *et al.*, 2015), which operates based on the WikiPathways platform (Kelder *et al.*, 2012; Kutmon *et al.*, 2016). Genes and Loci were sorted based on the p-value, from lowest to highest p-value, independent of lg2FC. Unique gene names annotated to the top 500 hits of these lists were used as test lists. Only pathways with a Z-score > 2 and enrichment of more than 3 genes were considered as enriched.

Results

Behavioural results

Anxiety-related behaviours

In the 3-CST, PS offspring spent less time in the chamber with the social target (U = 1078.0, p = 0.006). While no general effect of genotype was observed, an overall group difference ($\chi^2(3) = 8.4$, p = 0.038) revealed that the PS effect was particularly apparent in *5-Htt*$^{+/-}$ offspring (U = 254.0, p = 0.012; Figure 1 A). Next to the effect of PS, a notable inter-individual difference was observed (Figure 1 B). A substantial part of the PS offspring displayed a performance indistinguishable from control offspring, while an almost equal number of animals showed a significantly lower preference for the social target. Furthermore, based on a cut-off at chance level (for detail see methods) we identified a group of resilient animals, with an overall resilience effect ($\chi^2(2) = 55.0$, p < 0.001), Susceptible offspring spent less time with the social target than control or resilient offspring (p < 0.001 for all comparisons Mann-Whitney U; Figure 1 C).

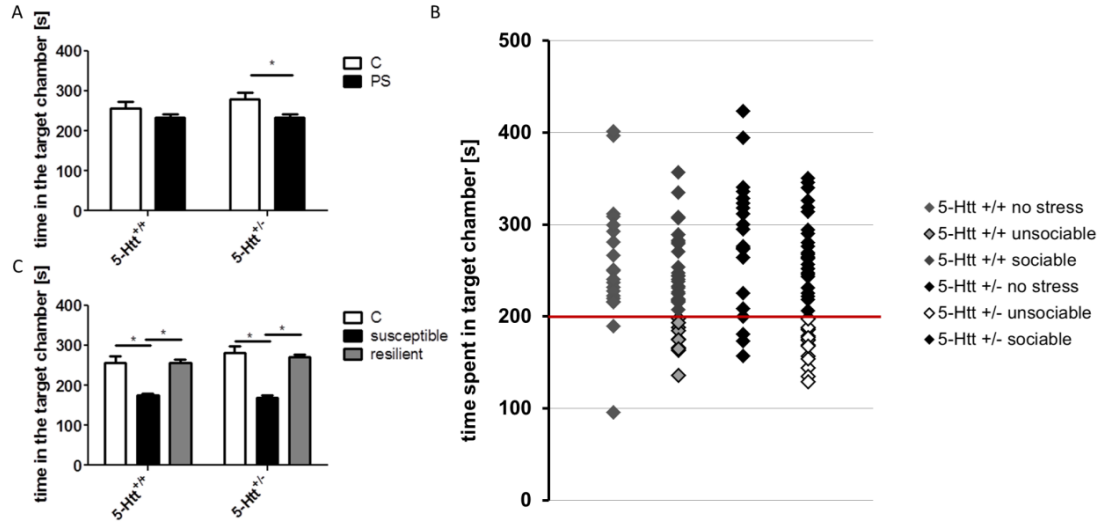

Figure 1 Social preference in the 3-chamber sociability test. (A) Time [s] spent with the social target. Overall, prenatal stress (PS) was associated with a significant reduction in sociability (p=0.006). This effect was, dependent on the gene variant of the serotonin transporter (*5-Htt*), as it was observable, in particular, in *5-Htt*$^{+/-}$ offspring (p = 0.012). (B) Inter-individual variability in time [s] spent with the social target among the various groups. (C) Time [s] spent with the social target when subdividing PS offspring in socially normal, i.e. resilient, and socially anxious, i.e. susceptible animals (based on a threshold of 200 s as displayed by the red line in panel B; overall effect of differential susceptibility: p < 0.001; see text for more details). Bars represent group means ± standard error, n = 10-26. *p < 0.050 (post-hoc Mann-Whitney U test).

In the EPM, differential susceptibility to PS affected both time spent ($\chi^2(5) = 14.2$, p = 0.014) and distance covered ($\chi^2(5) = 15.8$, p = 0.008) on the open arms of the maze, dependent on the *5-Htt* genotype (Figure 2). Susceptible *5-Htt*$^{+/+}$ offspring spent more time (U = 38, p = 0.009) and covered a greater distance (U = 35, p = 0.006) on the open arms, when compared to control offspring. In *5-Htt*$^{+/-}$ animals, in contrast, resilient offspring spent more time (U = 150, p = 0.026) and covered a greater distance (U = 138, p = 0.012) on the open arms when compared to control offspring. Time and distance in the open arms was furthermore increased in susceptible compared to resilient *5-Htt*$^{+/+}$ offspring (p <

0.050), but not in heterozygous resilient *5-Htt*$^{+/-}$ offspring when compared to susceptible *5-Htt*$^{+/-}$ offspring. The number of entries onto the open arm did not differ between groups. Correlation analysis revealed a negative correlation between the time spent in the chamber with the social target and time spent (ρ = -0.521, $p < 0.001$) and distance covered (ρ = -0.478, $p < 0.001$) in the open arm of the EPM in *5-Htt*$^{+/+}$ offspring. No correlation was found in *5-Htt*$^{+/-}$ offspring.

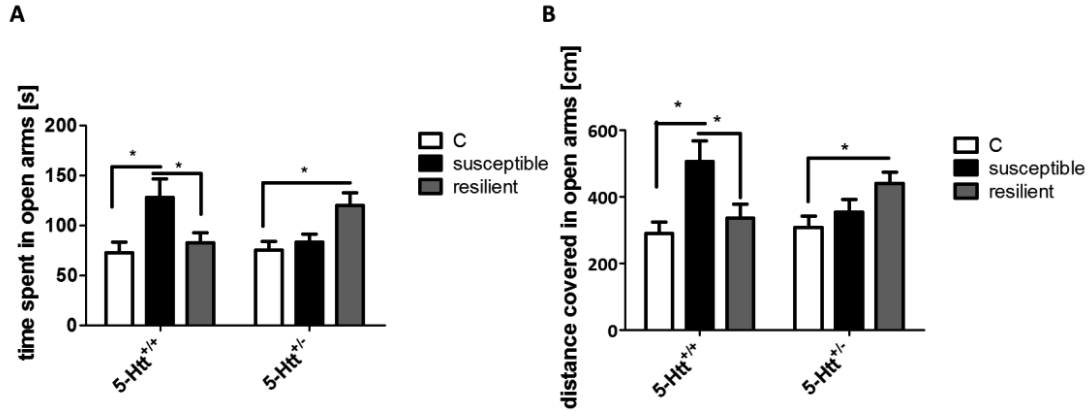

Figure 2 General anxiety in the elevated plus maze. Differential susceptibility towards prenatal stress (PS) affected anxiety in a serotonin transporter (*5-Htt*) gene variant-dependent fashion ($p < 0.020$). (A) Time spent [s] and (B) distance covered [cm] were comparably increased in susceptible *5-Htt*$^{+/+}$ ($p < 0.010$) and resilient *5-Htt*$^{+/-}$ ($p < 0.030$) offspring. Bars represent group means ± standard error, n = 10-26. *$p < 0.050$ (post-hoc Mann-Whitney U test).

Depressive-like behaviours

In the PST, *5-Htt*$^{+/+}$ offspring covered a smaller distance during the first 2 min (U = 1343.0, p = 0.034) when compared to *5-Htt*$^{+/-}$ offspring, independent of susceptibility or PS. This effect was gradually extenuated in the course of the 10 min test period (Figure 3). We did not observe any differences in sucrose preference.

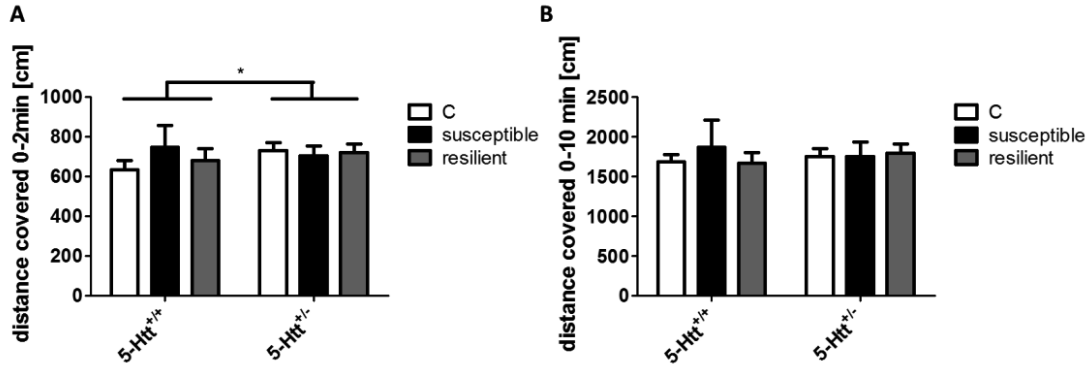

Figure 3 Depression-related behaviour in the Porsolt swim test. Serotonin transporter (*5-Htt*) deficiency increased the distance covered [cm] (A) during the first 2 min of the Porsolt swim test (PST), independent of differential susceptibility ($p < 0.04$). (B) After 10 min the effect was no longer observable. Bars represent group means ± standard error, n = 10-26. *$p < 0.050$ (post-hoc Mann-Whitney U test).

Physiological measures

At P35 body-weight was affected by PS, dependent on the genotype and the observed susceptibility ($\chi^2(5) = 11.7$, $p = 0.039$). In *5-Htt*$^{+/+}$ animals, PS decreased the weight, independent of the observed susceptibility ($p<0.040$), while in *5-Htt*$^{+/-}$ animals only susceptible animals showed a decrease in weight ($U = 92.5$, $p = 0.031$). The body-weight did not differ at later time-points. Furthermore, differential susceptibility was related with a *5-Htt* genotype-dependent difference in the total consumption (water and sucrose combined; $\chi^2(5) = 15.5$, $p = 0.009$). *5-Htt*$^{+/+}$ susceptible offspring drank more, compared to control ($U = 40.0$, $p = 0.025$) and resilient ($U = 64.0$, $p = 0.045$) offspring of the same genotype and *5-Htt*$^{+/-}$ PS offspring drank less compared to their *5-Htt*$^{+/+}$ counterparts ($p < 0.040$). Furthermore, corticosterone levels showed a notable increase following restraint stress ($F(1, 68) = 568.9$, $p < 0.001$, Greenhouse-Geisser) but were not significantly different between groups. Post-stress corticosterone was affected by differential susceptibility ($F(2,68) = 3.8$, $p = 0.026$), with lower corticosterone levels in resilient, compared to susceptible offspring ($p = 0.032$; Table 1).

Table 1 Plasma corticosterone response.

	corticosterone baseline			corticosterone stress *		
	C	**susceptible**	**resilient**	**C**	**susceptible**	**resilient**
***5-Htt*$^{+/+}$**	130.3± 17.5	139.3± 28.0	152.3± 17.9	634.2± 16.1	650.8± 18.7	606.1± 16.7
***5-Htt*$^{+/-}$**	121.2± 19.8	187.8± 35.8	124.0± 11.1	604.8± 17.7	639.6± 29.7	577.2± 15.0

Following 20 min restraint stress plasma corticosterone levels [ng/ml] were lower in resilient offspring when compared to susceptible offspring ($p = 0.030$), independent of the serotonin transporter (*5-Htt*) genotype. Values represent means ± standard error, n = 10-26.

Gene expression

Dependent on the *5-Htt* genotype, differential susceptibility was associated with an altered gene expression profile (S 8). In *5-Htt*$^{+/+}$ susceptible offspring, 22 DEGs, of which 14 were up-regulated and 8 were down-regulated, when compared to offspring of the control group, were found. In resilient offspring, of the same genotype, 13 genes were differentially expressed. Of these genes, 7 were up- and 6 down-regulated in their expression when compared to animals of the complementary control group (Figure 4 A). In *5-Htt*$^{+/-}$ offspring, susceptibility to PS altered the expression of 200 genes, of which 87 were higher and 113 were lower expressed than in control offspring. Resilience, in offspring of the same genotype, was associated with in total 334 DEGs. 102 of these DEGs were higher and 232 were lower expressed in the resilient offspring compared to controls (Figure 4 B). 65 DEGs were overlapping between susceptible and resilient *5-Htt*$^{+/-}$ offspring, none were overlapping between susceptible and resilient *5-Htt*$^{+/+}$ offspring.

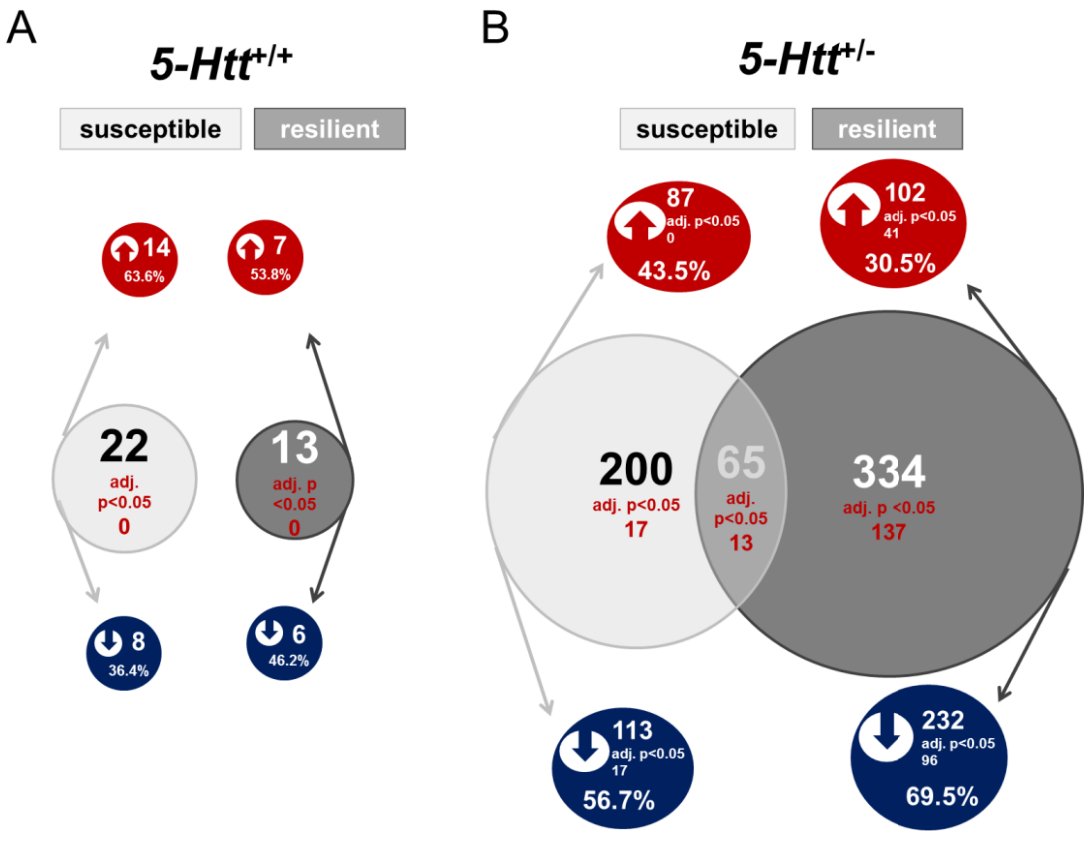

Figure 4 Number of differentially expressed genes in the hippocampus of susceptible and resilient offspring, dependent on the serotonin transporter gene variant. Gene expression was altered in a notably higher scale in serotonin transporter (*5-Htt*) -deficient offspring (B) than in *5-Htt*$^{+/+}$ offspring (A). Furthermore, directionality of observed changes was dependent on *5-Htt* genotype, with an overproportional number of up-regulated (red) differentially expressed genes (DEGs) in susceptible *5-Htt*$^{+/+}$ offspring (63.6%) and an overproportional down-regulation (blue) of DEGs in resilient *5-Htt*$^{+/-}$ offspring (69.5%). Data based on sequencing counts, n = 8 per group. DEGs were determined by a nominal p-value < 0.010 and log2 fold change > |0.2|.

Enriched annotated pathways, determined using the pathway analysis tool PathVisio (van Iersel *et al.*, 2008; Kutmon *et al.*, 2015) are depicted in Table 2. Overall, less pathways were affected in susceptible compared to resilient offspring and in *5-Htt*$^{+/+}$ compared to *5-Htt*$^{+/-}$ offspring.

Table 2 Enriched pathways based on the the top 500 hits of the DESeq2 analysis for differential gene expression.

Pathway	positive	measured	total	% overlap	Z-score	pvalue	genes
***5-Htt*$^{+/+}$ SUS-control**							
TYROBP Causal Network	5	58	60	8.62	4.29	1.00E-03	**LYL1, NPC2; IL10RA; C3; ZFP3612**
***5-Htt*$^{+/+}$ RES-control**							
G1 to S cell cycle control	4	61	63	6.56	4.19	3.00E-03	**CDKN2B;CDKN1A;CCND2;CCNG2**
PPAR signalling pathway	4	81	94	4.94	3.41	5.00E-03	**SCD4; FABP7;UCP1;RXRG**
***5-Htt*$^{+/-}$ SUS-control**							
***5-Htt*$^{+/-}$ RES-control**							
Exercise-induced Circadian Regulation	6	49	50	12.24	4.61	0.00E+00	**DNAJA1;PER1;UGP2;SUMO1;ZFR;TUBB4A**
Exercise-induced Circadian Regulation	5	48	49	10.42	3.72	2.00E-03	**DNAJA1;PER1;UGP2;SUMO1;ZFR**
Alpha6-Beta4 Integrin Signalling Pathway	6	67	68	8.96	3.61	0.00E+00	**RTKN;LAMB2;ITGB4;ABL1;LAMC1;PLEC**
Delta-Notch Signalling Pathway	6	83	85	7.23	2.97	9.00E-03	**NFKBIA;LFNG;HDAC2;SNW1;NOTCH4;ADAM10**
Notch Signalling Pathway	4	46	47	8.70	2.87	1.00E-02	**LFNG;HDAC2;SNW1;NOTCH4**
PodNet: protein-protein interactions in the podocyte	13	315	316	4.13	2.17	3.00E-02	**DDR1;EFNB1;KIRREL2;LAMB2;ITGB4;NOTCH4;CAPZA2; CDC42; TENC1; ADAM10;ACTN4;LAMC1;BCAR1**
XPodNet - protein-protein interactions in the podocyte expanded by STRING	28	827	837	3.39	2.17	3.30E-02	**EPHB6;DDR1; RTKN;ITGB4;NOTCH4;LAMC1;TOB2;PTPRF;EFNB1;SUMO1;CHN2;ABL1;KIRREL2;SPHK 2;LAMB2;ADAM10; OLIG1;ACTN4;GADD45G;RNF123;CAPZA2;CNTN2;BCAR1;SKAP2;PLEC; Cdc42; HDAC1; TENC1**

Pathway enrichment analysis, using PathVisio revealed several pathways to be affected by increased susceptibility (SUS) or resilience (RES), dependent on the serotonin transporter (*5-Htt*) genotype. Expression was assessed using RNA sequencing, results are based on RNA counts.

H3K4 tri-methylation

At the level of epigenetic regulation, H3K4me3 DELs were found for differential susceptibility to PS, dependent on the *5-Htt* genotype (S 8). Less differential enrichment was observed in *5-Htt*$^{+/-}$ resilient offspring, when compared to *5-Htt*$^{+/+}$ resilient offspring. In resilient *5-Htt*$^{+/+}$ offspring 703 loci were differentially enriched. Of these 128 were up- and 575 were down-regulated when compared to controls (Figure 5 A). In susceptible *5-Htt*$^{+/+}$ offspring, 62 fragments were differentially enriched, when compared to control offspring. Of these 25 were more and 37 less abundant in susceptible *5-Htt*$^{+/+}$ offspring. In *5-Htt*$^{+/-}$ offspring, 158 DELs were associated with susceptibility. Of these 65 were more and 93 less abundant in *5-Htt*$^{+/-}$ susceptible compared to control offspring. Resilient animals of the same genotype had 190 DELs, of which 89 DELs were up- and 101 down-regulated compared to controls (Figure 5 B).

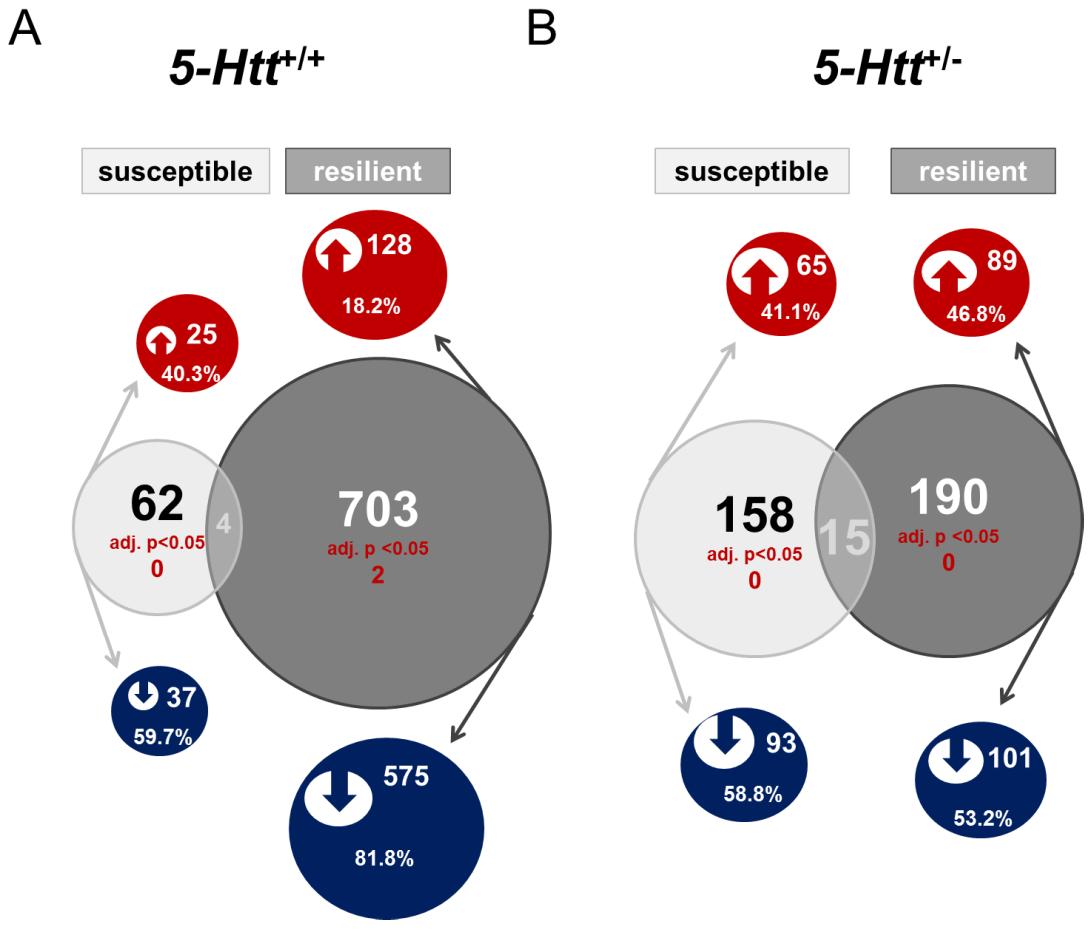

Figure 5 Number of differentially enriched loci, based on H3K4me3 immunoprecipitation in the hippocampus of susceptible and resilient offspring, dependent on the serotonin transporter gene variant. Fragment enrichment was altered in a notably higher scale in serotonin transporter (*5-Htt*)-deficient offspring (B) than in *5-Htt*$^{+/+}$ offspring (A). Furthermore, directionality of observed changes was dependent on *5-Htt* genotype, with an overproportional number of down-regulated (blue) H3K4me3 levels in resilient *5-Htt*$^{+/+}$ offspring (81.8%). Data based on sequencing counts, n = 6 per group. Differentially enriched loci (DELs) were determined by a nominal p-value < 0.010 and log2 fold change > |0.2|.

Pathway enrichment analysis revealed a number of enriched pathways, dependent on differential susceptibility and *5-Htt* genotype (Table 3). Several pathways were enriched for multiple comparisons of enriched fragments. For example, “Wnt signalling” was found to be enriched in *5-Htt*$^{+/+}$ susceptible

and resilient offspring as well as in *5-Htt*$^{+/-}$ susceptible offspring. The “non-odorant GPCRs” pathway was found to be enriched for resilient animals of either *5-Htt* genotype. “TGFbeta signalling” was found to be enriched for the comparison of *5-Htt*$^{+/+}$ resilient offspring to controls and the *5-Htt*$^{+/-}$ susceptible offspring to controls. However, the number of overlapping DELs between comparisons was negligible for either of the overlapping pathways.

Table 3 Enriched pathways based on the top 500 hits of the DESeq2 analysis for differential enrichment of H3K4me3 loci.

Pathway with enriched terms	positive	measured	total	% overlap	Z-score	pvalue	genes
***5-Htt*$^{+/+}$ SUS-control**							
Adipogenesis genes	8	116	133	6.90	3.03	3.00E-03	NCOA1;BMP3;CYP26B1;MBNL1;STAT1;SPOCK1;GATA2;KLF15
Wnt Signalling Pathway	4	58	62	6.90	2.13	2.80E-02	PPP2R5E;WNT3A;FZD9;PRKD1
Kit Receptor Signalling Pathway	4	60	68	6.67	2.05	4.20E-02	GRB7; PTPRU;STAT1;KIT
***5-Htt*$^{+/+}$ RES-control**							
Wnt Signalling in Kidney Disease	8	35	39	22.86	5.83	0.00E+00	
ESC Pluripotency Pathways	14	108	124	12.96	4.94	0.00E+00	WNT2B;WNT3A;FZD9;WNT9B;FOS;WNT16;FGF3;WNT6;WNT11;AKT3;RAF1;BMPR1B;FGFR3;WNT3
Wnt Signalling Pathway	9	58	62	15.52	4.61	1.00E-03	FOSL1;WNT6;WNT11;WNT2B;WNT3A;FZD9;WNT16;PRKCZ;WNT3
Wnt Signalling Pathway and Pluripotency	11	91	98	12.09	4.09	0.00E+00	FOSL1;WNT6;WNT11;WNT2B;WNT3A;WNT9B;FZD9;WNT16;PRKCZ;NKD1;WNT3
Non-odorant GPCRs	16	190	267	8.42	3.31	0.00E+00	CCR1;VIPR2;PTGER2;PTGER3;FZD9;PTH2R;CELSR1;ADRA2B;ADRA2A;PROKR1;PROKR2;ADORA2B;GRM6;GALR1;BAI1;PRLHR
Wnt Signalling Pathway NetPath	10	100	110	10.00	3.20	6.00E-03	PIAS4;SMAD3;WNT3A;MAGI3;MYB;FZD9;CDK1;RAF1;NKD1;WNT3
SIDS Susceptibility Pathways	6	48	66	12.50	3.10	3.00E-03	VIPR2;FEV;HES1;LMX1B;AVP;SCN5A
TGF Beta Signalling Pathway	5	47	53	10.64	2.41	2.10E-02	SMAD2;ZEB2;SMAD3;FST;FOS
Signalling of Hepatocyte Growth Factor Receptor	4	34	35	11.76	2.39	2.30E-02	MAP4K1;PIK3CA;FOS;RAF1
GPCRs, Other	5	55	210	9.09	2.01	4.10E-02	GPR73; CELSR1; IRX6; PTHR2; FZD9
***5-Htt*$^{+/-}$ SUS-control**							
MicroRNAs in Cardiomyocyte Hypertrophy	7	78	109	8.97	3.57	4.00E-03	HDAC4;miR125b; DVL1;AKT1;PRKG1;NFKB1;MAP2K6
TGF Beta Signalling Pathway	5	47	53	10.64	3.48	6.00E-03	CREBBP;SMAD4;FST;LEF1;NFKB1
Notch Signalling Pathway	4	39	47	10.26	3.02	1.00E-02	CREBBP;KAT2A;NOTCH4;DVL1
PluriNetWork	14	254	292	5.51	3.01	4.00E-03	ACVR1;NCOA1;HDAC4;CREBBP;SMAD4;ZFP57;LEF1;MBD2;DVL1;AKT1;WWP2;NFKB1;DAZL; 1600029D21Rik;
Wnt Signalling Pathway NetPath	7	100	110	7.00	2.80	9.00E-03	SMAD4;MAGI3;LEF1;MYB;DVL1;AKT1;TCF4
Exercise-induced Circadian Regulation	4	45	49	8.89	2.67	1.40E-02	PER1;PPP2CB;NCOA4; QK

Exercise-induced Circadian Regulation	4	47	50	8.51	2.56	1.90E-02	PER1;PPP2CB;NCOA4; QK
Myometrial Relaxation and Contraction Pathways	8	139	162	5.76	2.38	2.00E-02	ATF1;GSTO1;PRKACB;RGS9;PLCD1;NFKB1;SLC8A1;ATF4
IL-6 signalling Pathway	6	93	100	6.45	2.36	2.30E-02	NCOA1;PPP2CB;CREBBP;AKT1;NFKB1;MAP2K6
Delta-Notch Signalling Pathway	5	74	85	6.76	2.27	2.80E-02	SMAD4;NOTCH4;LEF1;AKT1;FURIN
***5-Htt*$^{+/-}$ RES-control**							
Monoamine GPCRs	5	29	41	17.24	4.78	1.00E-03	HRH1;CHRM4;ADRA2B;DRD5
Non-odorant GPCRs	11	190	267	5.79	2.61	5.00E-03	DRD1A; GPRC5B;HRH1;GRM5;CCKBR;P2RY14;CHRM4;ADRA2B;GRM1;CRHR2;DRD5
Endochondral Ossification	4	54	68	7.41	2.10	3.30E-02	CDKN1C;TGFB2;PLAU;PLAT

Pathway analysis using Pathvisio revealed several pathways to be affected by increased susceptibility (SUS) or resilience (RES), dependent on the serotonin transporter (*5-Htt*) genotype. H3K4me3 enrichmant was assessed using chromatin immunoprecipitation (ChIP)-based sequencing, results are based on peak counts.

Related gene expression and H3K4me3 enrichment

Next, to determine the extent to which DELs and DEGs were related, DEG and DEL data of the hippocampal gene expression and H3K4me3 profiles of the same mice, as previously reported, were compared. In *5-Htt*$^{+/+}$ susceptible and resilient offspring, gene expression and H3K4me3 profile did not overlap. In *5-Htt*$^{+/-}$ susceptible offspring fatty acid binding protein 7 (*Fabp7*) and neurexophilin and PC-esterase domain family member 2 (*Nxpe2*) were found to be differentially expressed and enriched for H3K4me3. In *5-Htt*$^{+/-}$ resilient offspring, period circadian clock 1 (*Per1*), solute carrier family 6 member 9 (*Slc6a9*), troponin I1 slow skeletal type (*Tnni1*), oligodendrocyte (OL) transcription factor 1 (*Olig1*), hyaluronan and proteoglycan link protein 2 (*Hapln2*), gap junction protein gamma 2 (*Gjc2*), gap junction protein beta 1 (*Gjb1*) and predicted gene *Gm14372* were differentially expressed and showed a differential H3K4me3 profile. None of the affected DEL pathways showed an overlap with pathways identified for RNA expression.

Discussion

The aim of the current study was to investigate differential susceptibility towards early adversity in the context of genetic variation of 5-HTT. Behavioural screening and genome-wide profiling of gene expression and H3K4me3 revealed that differential susceptibility to PS was associated with a distinct behavioural profile as well as numerous molecular changes. Most observed changes were dependent on the *5-Htt* genotype, with a notable discrepancy in the number of regulated DEGs and a similar, but more moderate difference in the number of DELs.

In a previous study, using the same maternal restraint stress paradigm of PS, the behavioural effects of PS seemed to be particularly pronounced in *5-Htt*$^{+/-}$ offspring (van den Hove *et al.*, 2011), while the same study highlighted that not all offspring responded to PS exposure to the same degree (Jakob *et al.*, 2014). A subset of PS offspring showed a PST performance comparable to control offspring, which was independent of the *5-Htt* genotype. Subsequent molecular analyses, in both studies, determined that observed changes in gene expression, associated with either PS effects per se (van den Hove *et al.*, 2011) or, more specifically, differential susceptibility to PS (Jakob *et al.*, 2014), were dependent on the *5-Htt* genotype of the investigated offspring. In the present study, similar effects of PS on anxiety-related behaviours were observed. A substantial part of the PS offspring showed a performance indistinguishable from control offspring, whereas an almost equal number of animals showed a clearly altered behavioural profile. Interestingly, this observed distinction between offspring that seemed unaffected and affected offspring was both context- (i.e. test-) and genotype-specific. In susceptible *5-Htt*$^{+/+}$ PS offspring, a decrease in anxiety-related behaviours in the EPM compared to control offspring was observed, whereas *5-Htt*$^{+/+}$ resilient PS offspring displayed anxiety-levels comparable to the levels observed in control offspring. In *5-Htt*$^{+/-}$ offspring, this relation was inverted and resilient and susceptible PS offspring displayed decreased or normal anxiety in the EPM, respectively. In contrast to the previous study, all observed effects of PS were exclusively seen at the level of anxiety-related behaviours, while no effects of PS on depression-related behaviours were observed. This might be explained by the fact that in the previous study *5-Htt*$^{+/-}$ dams were used, while in this study the mothers were wildtype (WT) mice, thus providing a differential prenatal environment. In line with this hypothesis, a recent investigation by Jones and co-workers showed that offspring of both *5-Htt*$^{+/-}$ and *5-Htt*$^{+/+}$ dams

that were stressed during pregnancy, showed signs of general decreased anxiety in the EPM, while increased social anxiety was dependent on the maternal genotype, with the major effect of PS being induced in the offspring of PS *5-Htt*$^{+/-}$ dams (Jones *et al.*, 2010).

Generally, PS in rodents is associated with a multitude of behavioural changes, such as increased anxiety (Schulz *et al.*, 2011; Zohar and Weinstock, 2011; Hiroi *et al.*, 2016), altered social behaviour (Miyagawa *et al.*, 2011; Schulz *et al.*, 2011) and increased depressive-like behaviour (Alonso *et al.*, 1991; Hiroi *et al.*, 2016). Interestingly, these behavioural observations were associated with changes in 5-HT system functioning, albeit the 5-HT-related changes were not consistent over experiments. Animals, exposed to PS, were found to show an increased immunoreactivity of 5-HT and tryptophan hydroxylase (TPH) in the dorsal raphe (Miyagawa *et al.*, 2011), a decrease in 5-HT immunoreactivity in the dorsal raphe and an increase in TPH2 immunoreactivity in the hippocampus (van den Hove *et al.*, 2014), a decrease in 5-HT1a receptor binding in both male and female offspring (Van den Hove *et al.*, 2006), an increased *Tph2* mRNA expression, selectively in female offspring, in the caudal dorsal raphe at P7, and a decrease of *Tph2* mRNA expression in adulthood (Hiroi *et al.*, 2016). In line with the current study, these data support the notion of a 5-HT-dependent programming of brain development in response to early-life stress.

To further investigate the molecular mechanisms underlying differential susceptibility to PS in the context of variation in the *5-Htt* gene in the current study, genome-wide gene expression and H3K4me3 profiling was performed on hippocampal tissue of the behaviourally screened mice. The hippocampus is one of the major regulators of stress responsivity via the HPA axis (Herman *et al.*, 2005). It is densely innervated by 5-HT projections from the B8 raphe nucleus (Muzerelle *et al.*, 2016) and all subtypes of 5-HT receptors were found to be expressed in the hippocampus (Berumen *et al.*, 2012). Dysregulation of the 5-HT system, as observed in PS, or through genetic variation, or by a combination of both, could therefore lead to an alteration in its development and functioning. In line with this, the *5-Htt* genotype seemed to introduce a prominent difference, with up to 30-fold more DEGs in *5-Htt*$^{+/-}$ offspring when compared to their wildtype counterparts. In contrast, three-fold more DELs were found to be associated with *5-Htt*$^{+/+}$ differential susceptibility. Overall, resilience was found to involve up to 334 DEGs and 703 DELs, dependent on offspring *5-Htt* genotype. Notably fewer genes were regulated in susceptible offspring. Next to the number, the directionality of the observed changes showed a remarkable pattern with more than 50% of RNA expression changes in *5-Htt*$^{+/+}$ offspring showing an upregulation, while in susceptible *5-Htt*$^{+/-}$ offspring only 43% and in resilient *5-Htt*$^{+/-}$ offspring only 30.5% of the changed genes were expressed to a higher extent. With regard to H3K4me3 enrichment, all changes showed an overrepresentation of a negative directionality, i.e. less H3K4me3 in susceptible as well as resilient offspring of either genotype. In the light of a strong association of H3K4me3 and active gene transcription, this observation is representing a regulatory discrepancy in *5-Htt*$^{+/+}$ offspring, which is particularly apparent in resilient *5-Htt*$^{+/+}$ offspring and might explain the general decrease in the number of DEGs in *5-Htt*$^{+/+}$ offspring. Overall, in this genotype, the observed discrepancy in directionality, between expression and H3K4me3 profile, suggests additional levels of regulation. The idea of multi-level regulation is not new and complex interactions between histone modifications, DNA methylation and other transcriptional regulators, such as DNA binding factors, were observed to exert their effects in concert, altering and/or maintaining a more or less accessible chromatin state (Jenuwein and Allis,

2001; Narlikar, Fan and Kingston, 2002; Barski *et al.*, 2007; Garske *et al.*, 2010; Kratz *et al.*, 2010; Fuchs *et al.*, 2011). In the current study, DNA methylation might represent an additional layer of a higher-order, complex, regulatory system, besides the investigated H3K4me3 histone mark. DNA methylation has been observed to provide a priming effect, next to directly affecting chromatin. Such priming might affect the regulatory sites in a way that renders them more or less receptive towards additional regulation by other histone-modifications or transcription factors (Liu *et al.*, 2016). Similarly, H3K4me3 could represent the priming mechanism. As such, H3K4me3 histone mark was shown to inhibit the interaction of DNA methyl transferase 3 (DNMT3) with DNA (Ooi *et al.*, 2007) and is often enriched at CpG islands (Thomson *et al.*, 2010). Furthermore, H3K4me3 is mostly associated with transcription start sites (Barski *et al.*, 2007). It has to be mentioned that regarding the determination of DELs, the mapping efficiencies of some of the samples were lower than expected and might introduce a sample-dependent bias, even though no group-specific or experimental effect on mapping efficiency was observed. Data should be **interpreted with this in mind** and potential candidates should be validated and further investigated with regard to other regulatory markers such as DNA methylation.

In the context of earl-life-induced, epigenetic programming, the 5-HT system has been suggested to be critically involved in epigenetic mechanisms, the development of the brain, and associated behavioural phenotypes (Homberg and van den Hove, 2012). One mechanism of such early-life programming was shown to be dependent upon increased 5-HT turnover in the hippocampus (Smythe, Rowe and Meaney, 1994). Alternating levels of maternal care had been shown to trigger a cascade of processes, resulting in an altered epigenetic state and expression of multiple genomic regions (Zhang *et al.*, 2013). This mechanism was proven to be, at least in part, dependent on the availability of 5-HT (Hellstrom *et al.*, 2012). Moreover, the observed changes comprised both DNA methylation changes as well as changes in histone acetylation (H3K9ac) and methylation (H3K4me3), the latter two of which showed an opposing directionality (Zhang *et al.*, 2013). In another study, chronic restraint stress that lasted for 21 days increased H3K4me3 in the dentate gyrus of adult rats. Fluoxetine treatment during stress had no effect on H3K4me3 levels (Hunter *et al.*, 2009). However, another histone mark, H3K9me3 that was found to be decreased by the stress paradigm was affected by fluoxetine treatment. Interestingly, while H3K4me3 is associated with active promoters, H3K9me3 has been related more to heterochromatin (Barski *et al.*, 2007) and, thus, might provide a 5-HT-specific regulation of specific gene clusters. In the current study, variation in *5-Htt* genotype was associated with prominent differences at the transcriptomic and epigenetic level. Amongst the DEGs, several epigenetic regulatory genes were observed. For example, serum/glucocorticoid regulated kinase 1 (*Sgk1*), which has been associated with glucocorticoid receptor (GR) activity (Bockmühl *et al.*, 2015), displayed decreased expression in both susceptible and resilient *5-Htt*$^{+/-}$ PS offspring. The decrease was more pronounced in susceptible offspring. GRs were shown to influence DNA methylation throughout development (Thomassin *et al.*, 2001; Niwa *et al.*, 2013; Bose *et al.*, 2015). Moreover, nerve growth factor-induced protein A (NGFI-A; also known as EGR1), involved in epigenetic programming of GR expression (Weaver *et al.*, 2007), was only decreased in expression in susceptible *5-Htt*$^{+/-}$ offspring and dual specificity phosphatase 1 (*Dusp1*), also associated with GR activity (Bockmühl *et al.*, 2015), was only down-regulated in resilient *5-Htt*$^{+/-}$ offspring when compared to control *5-Htt*$^{+/-}$ offspring. Overall, this is suggesting distinct gene expression profiles in *5-Htt*$^{+/-}$ offspring, relating early adversity to the expression of epigenetic effectors.

Moreover, in accordance with the previously discussed discrepancy between the number of DEGs and DELs, only a marginal number of DELs were found to overlap with DEGs. In *5-Htt*$^{+/+}$ offspring, no overlap between gene expression and H3K4me3 enrichment was observed, while in *5-Htt*$^{+/-}$ offspring 10 DEGs displayed concomitant H3K4me3 enrichment. In susceptible *5-Htt*$^{+/-}$ offspring, *Fabp7* showed both, an increase in expression and H3K4me3 enrichment. Furthermore, the expression of this gene was found to be down-regulated in resilient *5-Htt*$^{+/+}$ offspring in an opposing direction. *Fabp7* expression can be observed throughput the whole life and has a peak at E14, when it is expressed by neural progenitor cells (Matsumata, Inada and Osumi, 2016). In adulthood, *Fabp7* was found to be expressed by astrocytes within the amygdala. Furthermore, FABP7 is suggested to be involved in proliferation, survival and differentiation of new born neural cells in the hippocampus, consequently affecting cell-type composition (Matsumata, Inada and Osumi, 2016). In this respect, *Fabp7* has been suggested to be involved in manifold mental disorders, such as bipolar disorder and schizophrenia (Matsumata, Inada and Osumi, 2016). *Fabp7*-deficient mice need more time for fear-memory extinction and are more anxious, compared to WT mice (Owada *et al.*, 2006). Interestingly, in the current study, we observed an increase in expression in 5-HTT-deficient, susceptible offspring, thus, animals that showed unaltered anxiety, but impaired social behaviour following PS. It might be that, in interaction with the consequences of altered 5-HT signalling and PS, increased *Fabp7* expression leads to an unfavourable outcome. In resilient *5-Htt*$^{+/+}$ offspring the expression was decreased, independent of H3K4me3 regulation. These animals showed normal social and anxiety behaviour. This could indicate that the regulation of *Fabp7* via H3K4me3 is dependent on the *5-Htt* genotype and might represent a compensatory mechanism.

In resilient *5-Htt*$^{+/-}$ offspring, we identified another gene, *Olig1*, associated with basic brain morphology and OL development, to be altered both in expression and H3K4me3 enrichment. *Olig1* is a transcription factor involved in OL differentiation (Zhou, Wang and Anderson, 2000). This is in line with the notion of the critical role of hippocampal cell-type composition in mental health, mentioned above. Similar to the down-regulation of *Fabp7* in resilient *5-Htt*$^{+/+}$ offspring, the down regulation of *Olig1* could represent a compensatory mechanism, rescuing the social phenotype. Interestingly, stress or corticosterone exposure is able to increase oligodendrogenesis in the dentate gyrus of adult animals via the GR, which at the same time decreased neurogenesis (Chetty *et al.*, 2014). The down-regulation of myelin associated genes, in general, might therefore represent a compensatory mechanism, reinstating the equilibrium between neuronal and glial cells. Studies, investigating the effects of early adversity on OLs and myelination, found a decrease in area coverage with myelin basic protein (MBP) in the hippocampus of guinea pig pups, exposed to PS, which also induced increased anxiety in the open-field test (Bennett *et al.*, 2015), a decrease in MBP immunoreactivity in the cerebral hemispheres of neonatal rats at P14, which had been treated with the corticosteroid dexamethasone for 5 days after birth, in the context of apoptotic degeneration of OL progenitors at P5 (Kim, Kim and Chang, 2013), and precocious myelination in the basolateral amygdala of male mice, following early weaning (Ono *et al.*, 2008). Electron microscopy showed an increase in the number and a decrease in the diameter of myelinated axons in the anterior part of the basolateral amygdala in early-weaned male mice at 5 weeks of age, which was associated with increased anxiety in the EPM (Ono *et al.*, 2008). Next to early adversity, the 5-HT system was shown to be involved in myelin-associated regulation. The effect of altered 5-HT signalling on OLs was reported previously. For example, perinatal treatment with

selective 5-HT reuptake inhibitors (SSRIs) resulted in alterations of the myelination of callosal and somatosensory axons as well as interferences with OL soma morphology (Simpson *et al.*, 2011). Simpson and colleagues, furthermore, observed *in vitro* that treatment with concentrations of 5-HT in the range of 10–100 µM induces OL pathology, while no such effects were observed with low or no 5-HT exposure (Simpson *et al.*, 2011). Another study, investigating the interaction of 5-HT and myelination and OLs, showed that 5-HT receptor 1a and 2a subtypes were expressed in OL lineages and that 5-HT treatment-induced death of OLs was, at least in parts, mediated by the 5-HT2a receptor (Fan *et al.*, 2015). OLs that were co-cultured with neurons, did not die following 5-HT exposure. However, they showed that in these co-cultures, exposure to 5-HT induced aberrant patterns of contactin-associated protein clustering at the sites of Ranvier's nodes, which suggests that 5-HT exposure may affect other axon-derived factors for myelination and, thus, alter neural connectivity (Fan *et al.*, 2015). Interestingly, in the current study, we observed an overrepresented number of myelination- and OL-related genes to be down-regulated in expression in *5-Htt*$^{+/-}$ resilient offspring. Amongst these genes were, for example, *Mbp*, which was identified in a previous study as regulated by DNA methylation, dependent on the investigated 5-HTT-by-PS interaction of the heterozygous *5-Htt* gene variant and PS (van den Hove *et al.*, 2011; Schraut *et al.*, 2014). Other factors of myelination that were significantly changed in the current study in *5-Htt*$^{+/-}$ resilient offspring, were SRY (sex determining region Y)-box 10 (*Sox10*) and myelin regulatory factor (*Myrf*) that are known to be essential myelin transcription factors in the central nervous system, where they induce terminal OL differentiation (Hornig *et al.*, 2013). While *Sox10* is expressed already in OL progenitors, *Myrf* expression starts only in mature OLs, i.e. after cell-cycle exit. Furthermore, SOX10 directly induces *Myrf* expression and, subsequently, both factors were reported to cooperate in inducing the expression of a myelin-gene network (Hornig *et al.*, 2013). Moreover, myelin associated glycoprotein (*Mag*), which is involved in cell-cell, protein-protein interactions, and as such controlling cell type-specific neurite outgrowth and regeneration (Sedzik, Jastrzebski and Grandis, 2015), claudin11 (*Cldn11*), known to be involved in membrane interactions at tight junctions and with the extracellular matrix (Bronstein *et al.*, 2000) and fatty acid 2-hydroxylase (*Fa2h*) that is involved in myelin metabolism (Kota and Hama, 2014), were found to be differentially expressed. Taken together, these observations in PS offspring suggest that in resilient *5-Htt*$^{+/-}$ PS offspring oligodendrogenesis- and myelination-related factors are decreased. Thus, oligodendrogenesis might, at least in part, be regulated via H3K4me3 programming of *Olig1* expression. This effect is restricted to *5-Htt*$^{+/-}$ offspring, suggesting an alternative mechanism for programming resilience in *5-Htt*$^{+/+}$ PS offspring. This alternative programming might be related to regulating cell-type composition in the hippocampus. It has to be mentioned that none of the overlapping DEGs correlated significantly with H3K4me3 enrichment, which might be explained, as previously discussed, by a multi-layered, epigenetic regulation. Another possible explanation might be low sample size, cell-type composition or cell specific functioning and programming by H3K4me3 that could not be accounted for in the homogenate.

Next to the overlap of expression and H3K4me3 enrichment, functional annotation clustering of the top 500 hits of gene expression or H3K4me3 analysis allowed us to identify several pathways, related to *5-Htt* genotype-dependent differential susceptibility. No pathway was enriched for both RNA expression and histone methylation. However, we were able to identify an interesting pattern, as for example in *5-Htt*$^{+/-}$ offspring, PS resilient offspring showed an enrichment for "exercise induced

circadian regulation" with regard to expression, while in the complementary PS susceptible offspring this pathway was found enriched with regard to H3K4me3 enrichment. Furthermore, one gene in this pathway, *Per1*, which is a transcriptional repressor and as such a key component of the "circadian rhythm regulation" pathway (Panda, Hogenesch and Kay, 2002), was found to display differential expression and H3K4me3 enrichment in resilient *5-Htt*$^{+/-}$ PS offspring, while in susceptible *5-Htt*$^{+/-}$ offspring only H3K4me3 enrichment was affected. Similar to the pathway for "exercise induced circadian rhythm", "delta notch signalling" and "notch signalling" were found enriched at the level of expression in resilient and, at the level of H3K4me3 enrichment, in susceptible *5-Htt*$^{+/-}$ offspring. Notch signalling has been suggested to be critically involved in OL development (Hu *et al.*, 2006). Furthermore, we did not identify a single pathway, related to the gene expression changes, observed in susceptible *5-Htt*$^{+/-}$ offspring. In *5-Htt*$^{+/+}$ offspring, no such interaction of differential susceptibility and genotype were observed. Interestingly, though, we identified the "peroxisome proliferator-activated receptor (PPAR) pathway", which has been found to be involved in the *Fabp7* mediated effects on proliferation (Tripathi *et al.*, 2017), as enriched in DEGs in *5-Htt*$^{+/+}$, resilient offspring, supporting the notion discussed above that myelin might be a key-mediatory factor of susceptibility or resilience.

Overall, the current study showed that exposure to PS resulted in a notable decrease in social behaviour. This decrease in social behaviour amongst PS offspring was more pronounced in *5-Htt* $^{+/-}$ animals and showed a high inter-individual variability, suggesting that part of the offspring was resilient to PS. Moreover, this differential susceptibility was distinguished by *5-Htt* genotype-dependent, specific transcriptomic and epigenetic signatures, supporting the concept of 5-HT-dependent, developmental programming. The observed molecular changes were, in particular, related to genes associated with OL differentiation and myelination.

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Supplementary information

Afm and *Gapdh* as control primers for successful H3K4me3 chromatin immunoprecipitation

To determine the successful immunoprecipitation of the histone marker H3K4me3, which is very closely related to actively expressed genes, we chose as a positive control the glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), which is widely used as a reference gene for qPCR and excessively reported to be stably expressed in the brain. As a negative control we chose afamin (*Afm*), which is not expressed in the brain of adult mice. Both primer pairs were located in the area of the transcription start site and are displayed in S 1.

S 1 Table Primer designed for positive and negative control of successful, specific immunoprecipitation of H3K4me3.

Oligo Name	Sequence 5' to 3' (include modification codes if applicable)	Scale (µmole)	Purification	Company
mmu-gDNA-Gapdh F'	CTGGGCCTCTCTCATTTCCC	0.025	desalted	Sigma-Aldrich
mmu-gDNA-Gapdh R'	CTTGGTGCGTGCACATTTCA	0.025	desalted	Sigma-Aldrich
mmu_gDNA_AFM_F	CTAGCCCTACCCACAAAGCC	0.025	desalted	Sigma-Aldrich
mmu_gDNA_AFM_R	CCCCTAACTTAGCATTCCTGGT	0.025	desalted	Sigma-Aldrich

UCSC *In-Silico* PCR (https://genome.ucsc.edu/cgi-bin/hgPcr) using the reference genome Mouse July 2007 (NCBI37/mm9) Assembly and the temperature calculations were done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temperature came from Primer3 (Untergasser *et al.*, 2012).

Gapdh:

>chr6:125115771-125115841 71 bp

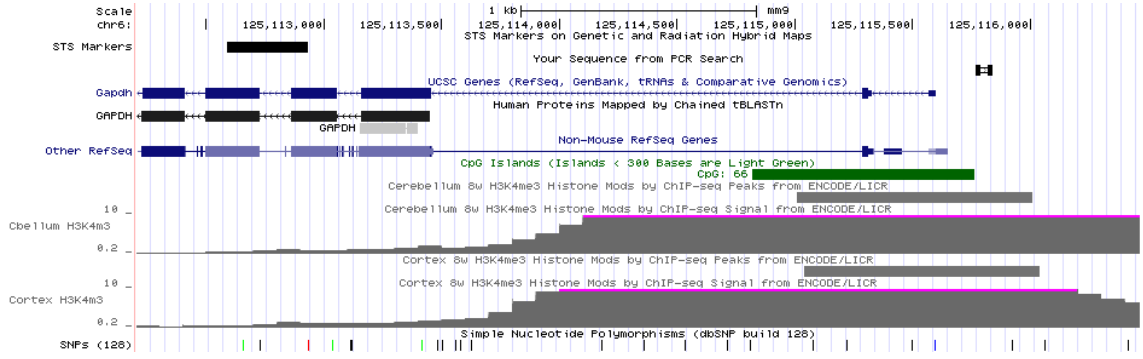
Primer Melting Temperatures of forward (63.3°C) ctgggcctctctcatttccc and reverse (64.2°C) cttggtgcgtgcacatttca primer.

The PCR product:

CTGGGCCTCTCTCATTTCCCctcctccctctctttggacccgcctcattttTGAAATGTGCACGCACCAAG

is displayed in S 2.

S 2 Figure Primer location at the glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) gene



Afm:

>chr5:90948075-90948146 72 bp

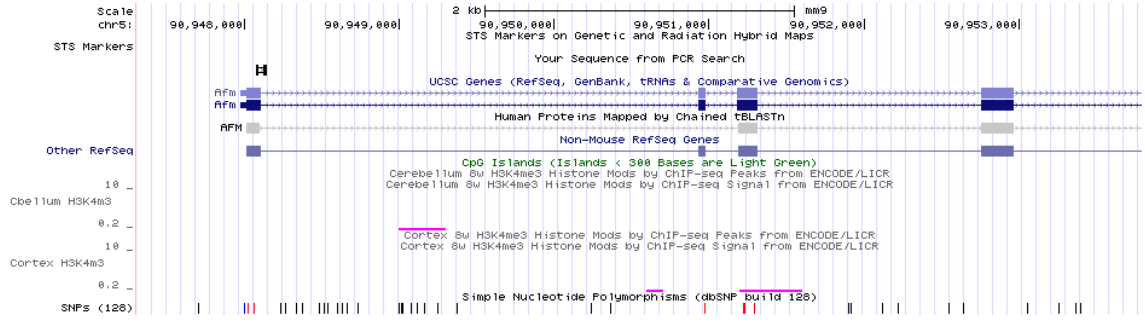
Primer Melting Temperatures of forward (61.0°C) ctagccctacccacaaagcc and reverse (60.7°C) cccctaacttagcattcctggt primer.

The PCR product:

CTAGCCCTACCCACAAAGCCtcaggacgtaggtaagtttacttgtgtattACCAGGAATGCTAAGTTAGGGG

is displayed in S 3.

S 3 Primer location at the afamin (*Afm*) gene



All samples were run in duplicate and the used fluorophore was SYBRgreen. QRT-PCR was performed on the LC4800, 384 well system (Roche, Basel, Switzerland). For one sample per group we used, in addition, an input control (IN) that was not subjected to immunoprecipitation as positive control and samples, immunoprecipitated with a mixture of random rabbit IgGs (RAB) (Vector Laboratories, Burlingame, CA, USA), as negative control. For the IN fraction, 100 µl were taken from the RAB samples. We provided 7 samples per group, of which the 6 best per group and each IN sample per group were sequenced (S 4).

S 4 Table Summary sequenced samples, n = 6 per group.

NXT-Dx No	Sample ID	Group	NXT-Dx No	Sample ID	Group	NXT-Dx No	Sample ID	Group
1	78	1	15	110	3	29	103	5
2	128	1	16	75	3	30	122IN	5
3	53IN	1	17	42	3	31	122	5
4	53	1	18	45	3	32	121	5
5	26	1	19	117	3	33	76	5
6	23	1	20	57IN	3	34	58	5
7	79	1	21	57	3	35	37	5
8	84	2	22	54IN	4	36	127IN	6
9	50	2	23	54	4	37	127	6
10	48	2	24	139	4	38	106	6
11	34	2	25	52	4	39	63	6
12	60IN	2	26	17	4	40	90	6
13	60	2	27	22	4	41	27	6
14	66	2	28	25	4	42	89	6

Results of the qRT-PCR reported in table S 5.

S 5 Table Positive and negative control for the specificity of the chromatin immuno precipitation using afamin (*Afm*) as negative and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) as positive control.

QC H3K4me3 ChIP											
Row	Column	Target	Sample	Plate-pos	Cp	**Row**	**Column**	**Target**	**Sample**	**Plate-pos**	**Cp**
A	9	AFM	17	A9	35.73	**A**	**1**	**GAPDH**	**17**	**A1**	**30.40**
A	10	AFM	17	A10	34.85	**A**	**2**	**GAPDH**	**17**	**A2**	**30.80**
C	9	AFM	22	C9	35.55	**C**	**1**	**GAPDH**	**22**		**32.60**
C	10	AFM	22	C10		**C**	**2**	**GAPDH**	**22**	**C2**	**30.74**
E	9	AFM	23	E9		**E**	**1**	**GAPDH**	**23**		**31.13**
E	10	AFM	23	E10		**E**	**2**	**GAPDH**	**23**	**E2**	**31.28**
G	9	AFM	25	G9		**G**	**1**	**GAPDH**	**25**		**31.60**
G	10	AFM	25	G10		**G**	**2**	**GAPDH**	**25**	**G2**	**31.55**
I	9	AFM	26	I9	35.64	**I**	**1**	**GAPDH**	**26**		**31.19**
I	10	AFM	26	I10		**I**	**2**	**GAPDH**	**26**	**I2**	**31.07**
K	9	AFM	27	K9		**K**	**1**	**GAPDH**	**27**		**30.45**
K	10	AFM	27	K10	35.8	**K**	**2**	**GAPDH**	**27**	**K2**	**30.72**
M	9	AFM	34	M9	34.71	**M**	**1**	**GAPDH**	**34**		**30.96**
M	10	AFM	34	M10		**M**	**2**	**GAPDH**	**34**	**M2**	**30.67**
O	9	AFM	35	O9	47.59	**O**	**1**	**GAPDH**	**35**		**-**
O	10	AFM	35	O10		**O**	**2**	**GAPDH**	**35**	**O2**	**-**
B	9	AFM	37	B9	35.72	**B**	**1**	**GAPDH**	**37**		**31.39**
B	10	AFM	37	B10	36.67	**B**	**2**	**GAPDH**	**37**	**B2**	**31.11**
D	9	AFM	42	D9	35.17	**D**	**1**	**GAPDH**	**42**		**31.54**
D	10	AFM	42	D10		**D**	**2**	**GAPDH**	**42**	**D2**	**31.54**
F	9	AFM	45	F9	34.8	**F**	**1**	**GAPDH**	**45**		**31.35**
F	10	AFM	45	F10	35.78	**F**	**2**	**GAPDH**	**45**	**F2**	**31.74**
H	9	AFM	48	H9	35.15	**H**	**1**	**GAPDH**	**48**		**30.34**
H	10	AFM	48	H10		**H**	**2**	**GAPDH**	**48**	**H2**	**30.33**
J	9	AFM	50	J9		**J**	**1**	**GAPDH**	**50**		**30.06**
J	10	AFM	50	J10	35.72	**J**	**2**	**GAPDH**	**50**	**J2**	**30.31**
L	9	AFM	52	L9		**L**	**1**	**GAPDH**	**52**		**30.13**
L	10	AFM	52	L10	34.72	**L**	**2**	**GAPDH**	**52**	**L2**	**29.75**
O	11	AFM	58	O11	40.71	**O**	**3**	**GAPDH**	**58**	**O3**	**32.61**
O	12	AFM	58	O12		**O**	**4**	**GAPDH**	**58**	**O4**	**32.62**
H	11	AFM	63	H11	40.55	**H**	**3**	**GAPDH**	**63**	**H3**	**32.00**
H	12	AFM	63	H12	35.59	**H**	**4**	**GAPDH**	**63**	**H4**	**31.69**
J	11	AFM	66	J11	35.84	**J**	**3**	**GAPDH**	**66**	**J3**	**30.30**
J	12	AFM	66	J12	36.46	**J**	**4**	**GAPDH**	**66**	**J4**	**30.03**
L	11	AFM	75	L11	35.66	**L**	**3**	**GAPDH**	**75**	**L3**	**31.82**
L	12	AFM	75	L12		**L**	**4**	**GAPDH**	**75**	**L4**	**33.10**
N	11	AFM	76	N11		**N**	**3**	**GAPDH**	**76**	**N3**	**33.62**
N	12	AFM	76	N12	37.15	**N**	**4**	**GAPDH**	**76**	**N4**	**33.40**
P	12	AFM	78	P12		**P**	**3**	**GAPDH**	**78**	**P3**	**30.88**
P	11	AFM	78	P11		**P**	**4**	**GAPDH**	**78**	**P4**	**30.69**
A	13	AFM	79	A13	34.87	**A**	**5**	**GAPDH**	**79**	**A5**	**30.97**
A	14	AFM	79	A14		**A**	**6**	**GAPDH**	**79**	**A6**	**30.79**
C	13	AFM	84	C13	42.08	**C**	**5**	**GAPDH**	**84**	**C5**	**30.71**

C	14	AFM	84	C14	35.55	**C**	**6**	**GAPDH**	**84**	**C6**	**30.72**
E	13	AFM	86	E13	34.85	**E**	**5**	**GAPDH**	**86**	**E5**	**33.65**
E	14	AFM	86	E14	35.86	**E**	**6**	**GAPDH**	**86**	**E6**	**32.78**
G	13	AFM	89	G13		**G**	**5**	**GAPDH**	**89**	**G5**	**30.77**
G	14	AFM	89	G14		**G**	**6**	**GAPDH**	**89**	**G6**	**30.18**
I	13	AFM	90	I13	34.53	**I**	**5**	**GAPDH**	**90**	**I5**	**31.51**
I	14	AFM	90	I14	35.07	**I**	**6**	**GAPDH**	**90**	**I6**	**31.9**
K	15	AFM	103	K15		**K**	**7**	**GAPDH**	**103**	**K7**	**30.61**
K	16	AFM	103	K16		**K**	**8**	**GAPDH**	**103**	**K8**	**30.62**
K	13	AFM	106	K13		**K**	**5**	**GAPDH**	**106**	**K5**	**31.83**
K	14	AFM	106	K14		**K**	**6**	**GAPDH**	**106**	**K6**	**31.51**
M	13	AFM	110	M13	45.88	**M**	**5**	**GAPDH**	**110**	**M5**	**32.45**
M	14	AFM	110	M14	36.91	**M**	**6**	**GAPDH**	**110**	**M6**	**34.39**
O	13	AFM	111	O13	35.19	**O**	**5**	**GAPDH**	**111**	**O5**	**32.77**
O	14	AFM	111	O14	33.92	**O**	**6**	**GAPDH**	**111**	**O6**	**32.65**
B	13	AFM	112	B13		**B**	**5**	**GAPDH**	**112**	**B5**	**37.05**
B	14	AFM	112	B14		**B**	**6**	**GAPDH**	**112**	**B6**	**35.59**
D	13	AFM	117	D13		**D**	**5**	**GAPDH**	**117**	**D5**	**32.77**
D	14	AFM	117	D14		**D**	**6**	**GAPDH**	**117**	**D6**	**33.13**
F	13	AFM	121	F13		**F**	**5**	**GAPDH**	**121**	**F5**	**31.41**
F	14	AFM	121	F14		**F**	**6**	**GAPDH**	**121**	**F6**	**31.23**
C	15	AFM	128	C15		**C**	**7**	**GAPDH**	**128**	**C7**	**32.1**
C	16	AFM	128	C16		**C**	**8**	**GAPDH**	**128**	**C8**	**31.22**
E	15	AFM	139	E15		**E**	**7**	**GAPDH**	**139**	**E7**	**33.00**
E	16	AFM	139	E16		**E**	**8**	**GAPDH**	**139**	**E8**	**32.99**
G	15	AFM	145	G15	35.18	**G**	**7**	**GAPDH**	**145**	**G7**	**32.94**
G	16	AFM	145	G16	46.87	**G**	**8**	**GAPDH**	**145**	**G8**	**33.22**
I	15	AFM	147	I15	47.65	**I**	**7**	**GAPDH**	**147**	**I7**	**34.36**
I	16	AFM	147	I16	34.54	**I**	**8**	**GAPDH**	**147**	**I8**	**33.34**
L	13	AFM	122 IN	L13	26.66	**L**	**5**	**GAPDH**	**122 IN**	**L5**	**27.85**
L	14	AFM	122 IN	L14	26.7	**L**	**6**	**GAPDH**	**122 IN**	**L6**	**27.87**
H	13	AFM	122 IP	H13	43.87	**H**	**5**	**GAPDH**	**122 IP**	**H5**	**30.68**
H	14	AFM	122 IP	H14		**H**	**6**	**GAPDH**	**122 IP**	**H6**	**30.97**
J	13	AFM	122 RAB	J13		**J**	**5**	**GAPDH**	**122 RAB**	**J5**	
J	14	AFM	122 RAB	J14	34.67	**J**	**6**	**GAPDH**	**122 RAB**	**J6**	
A	15	AFM	127 IN	A15	27.04	**A**	**7**	**GAPDH**	**127 IN**	**A7**	**28.56**
A	16	AFM	127 IN	A16	26.97	**A**	**8**	**GAPDH**	**127 IN**	**A8**	**28.46**
N	13	AFM	127 IP	N13		**N**	**5**	**GAPDH**	**127 IP**	**N5**	**32.22**
N	14	AFM	127 IP	N14		**N**	**6**	**GAPDH**	**127 IP**	**N6**	**31.36**
P	13	AFM	127 RAB	P13	36.77	**P**	**5**	**GAPDH**	**127 RAB**	**P5**	
P	14	AFM	127 RAB	P14	35.71	**P**	**6**	**GAPDH**	**127 RAB**	**P6**	
A	11	AFM	53 IN	A11	27.1	**A**	**3**	**GAPDH**	**53 IN**	**A3**	**28.42**
A	12	AFM	53 IN	A12	27.5	**A**	**4**	**GAPDH**	**53 IN**	**A4**	**28.62**
N	9	AFM	53 IP	N9		**N**	**1**	**GAPDH**	**53 IP**		
N	10	AFM	53 IP	N10		**N**	**2**	**GAPDH**	**53 IP**	**N2**	**31.47**
P	9	AFM	53 RAB	P9		**P**	**1**	**GAPDH**	**53 RAB**		
P	10	AFM	53 RAB	P10		**P**	**2**	**GAPDH**	**53 RAB**	**P2**	
G	11	AFM	54 IN	G11	27.33	**G**	**3**	**GAPDH**	**54 IN**	**G3**	**28.11**
G	12	AFM	54 IN	G12	26.65	**G**	**4**	**GAPDH**	**54 IN**	**G4**	**27.89**

C	11	AFM	54 IP	C11	35.95	**C**	**3**	**GAPDH**	**54 IP**	**C3**	**30.800**
C	12	AFM	54 IP	C12		**C**	**4**	**GAPDH**	**54 IP**	**C4**	**30.55**
E	11	AFM	54 RAB	E11	39.29	**E**	**3**	**GAPDH**	**54 RAB**	**E3**	
E	12	AFM	54 RAB	E12	35.71	**E**	**4**	**GAPDH**	**54 RAB**	**E4**	**37.75**
M	11	AFM	57 IN	M11	28.37	**M**	**3**	**GAPDH**	**57 IN**	**M3**	**29.53**
M	12	AFM	57 IN	M12	27.85	**M**	**4**	**GAPDH**	**57 IN**	**M4**	**29.3**
I	11	AFM	57 IP	I11		**I**	**3**	**GAPDH**	**57 IP**	**I3**	**30.27**
I	12	AFM	57 IP	I12	35.87	**I**	**4**	**GAPDH**	**57 IP**	**I4**	**29.96**
K	11	AFM	57 RAB	K11	42.73	**K**	**3**	**GAPDH**	**57RAB**	**K3**	**36.21**
K	12	AFM	57 RAB	K12		**K**	**4**	**GAPDH**	**57 RAB**	**K4**	
F	11	AFM	60 IN	F11	26.47	**F**	**3**	**GAPDH**	**60 IN**	**F3**	**27.63**
F	12	AFM	60 IN	F12	26.62	**F**	**4**	**GAPDH**	**60 IN**	**F4**	**27.74**
B	11	AFM	60 IP	B11	35.62	**B**	**3**	**GAPDH**	**60 IP**	**B3**	**30.06**
B	12	AFM	60 IP	B12	35.72	**B**	**4**	**GAPDH**	**60 IP**	**B4**	**29.74**
D	11	AFM	60 RAB	D11	33.59	**D**	**3**	**GAPDH**	**60 RAB**	**D3**	**34.43**
D	12	AFM	60 RAB	D12	34.11	**D**	**4**	**GAPDH**	**60 RAB**	**D4**	**35.26**
M	15	AFM	TE	M15		**M**	**7**	**GAPDH**	**TE**	**M7**	
M	16	AFM	TE	M16		**M**	**8**	**GAPDH**	**TE**	**M8**	

Mapping efficiency of the H3K4me3-based enrichment sequencing

Sequencing quality, GC content, overrepresented sequences and sequencing duplication levels were all in accordance with the expected thresholds, while mapping showed some deviations. The paired-end 50 bp sequencing reads were mapped using Bowtie2 (v2.3.2) software. S 6 summarizes the mapping statistics for all samples. As the mapping efficiencies of some of the samples were lower than expected, a couple of additional improvement steps were performed. During this process possible bias in unmapped or poorly mapped reads was assessed. However, no technical bias could be found both in data analysis steps or library prep. The results of this extra analysis suggest that the mapping efficiency bias is merely caused by the sample preparation (chromatin immunoprecipitation; ChIP) itself and the data should be **interpreted with this in mind**, as the coverage might differ between the individual samples.

S 6 Table Mapping efficiency of the sequencing using Bowtie.2

Sample	FL	TR	CR	Aligned [%]	Dup1 [%]	GC1 [%]	M Seq1	Dup2 [%]	GC2 [%]	M Seq2
17IP	232	0.21		30.5	13	46	32.7	11.4	46	32.7
22IP	221	0.18		31.7	11.9	47	22.5	10.7	48	22.5
23IP	232	0.22		51.8	10.4	50	13.2	9.7	50	13.2
25IP	232	0.28		54.8	18.3	53	22.9	15.9	53	22.9
26IP	233	0.18		43.9	8.5	50	10.1	7.8	50	10.1
27IP	224	0.21		41.6	10.9	48	15.6	10.2	48	15.6
34IP	235	0.20		42.3	9.9	48	15.8	8.9	48	15.8
37IP	234	0.22		50.6	11.3	49	16	10.5	49	16
42IP	220	0.25		56.9	13.4	52	15.3	12.4	52	15.3
45IP	220	0.22		49.9	12.1	49	16.4	11.4	49	16.4
48IP	224	0.23		57.3	14	50	15.8	12.3	50	15.8
50IP	232	0.19		48	9	49	10.8	8.3	49	10.8
52IP	226	0.24		78.9	15	51	24.3	13.3	51	24.3
53control	220	0.20	0.19							

53IN	71	0.19		37.4	11.6	45	26.4	10.3	45	26.4
53IP	220	0.20		62.2	11.6	49	12.9	10	49	12.9
54control	219	0.20	0.21							
54IN	73	0.21		46.5	12.3	44	23.8	11.8	45	23.8
54IP	219	0.20		59.1	12.3	49	22.6	11	49	22.6
57control	230	0.21	0.21							
57IN	70	0.21		35.6	10.8	45	23.5	10.4	45	23.5
57IP	230	0.21		54.8	11	50	18.7	10.3	50	18.7
58IP	213	0.19		30.1	10.7	47	15.2	10	47	15.2
60control	226	0.18	0.21							
60IN	65	0.21		30.3	10.8	45	20.7	10.4	46	20.7
60IP	226	0.18		51.7	10	48	15.1	9.5	48	15.1
63IP	210	0.22		33	11.3	45	14.1	10.4	45	14.1
66IP	229	0.18		43.2	10	48	15.9	9.5	48	15.9
75IP	216	0.21		51.2	11.2	50	14.6	10.4	50	14.6
76IP	222	0.28		53.5	17.7	54	13	16.5	54	13
78IP	217	0.20		56.5	9.5	51	13.1	8.6	51	13.1
79IP	239	0.22		78.4	11.7	52	11.7	10.9	52	11.7
84IP	233	0.21		65.7	10.2	52	11.3	9.5	52	11.3
89IP	232	0.27		58.1	13.3	51	15.8	12.6	51	15.8
90IP	208	0.22		36.8	11.9	46	19.5	11.2	46	19.5
103IP	216	0.21		51.1	13	50	23.9	11.6	50	23.9
106IP	212	0.25		49.5	13.1	49	19.7	11.9	49	19.7
110IP	201	0.18		36.1	10.3	45	18	9.6	45	18
117IP	214	0.22		40.4	12.8	49	16.9	12	49	16.9
121IP	221	0.28		57.8	16.3	52	23.4	14.6	52	23.4
122control	224	0.27	0.19							
122IN	69	0.19		38	11	45	26.2	10.2	45	26.2
122IP	224	0.27		75.8	17.2	54	21.4	15.2	54	21.4
127control	221	0.28	0.2							
127IN	68	0.20		32	11.1	45	26.9	10.8	45	26.9
127IP	221	0.28		68.1	17	53	15.2	15.6	53	15.2
128IP	215	0.23		57.4	10.9	50	14.9	10.3	50	14.9
139IP	230	0.20		44.5	12.8	49	20.1	11.3	49	20.1

The table shows the fragment length (FL), treatment redundancy (TR), control redundancy (CR), efficiency of the mapping (aligned [%]), furthermore the % of duplicates (Dup) and GC content [%] and total sequences in millions (M Seq) for reads 1 and 2 of the paired-end reads.

Dam weight and litter size

Dam weight was recorded on E0, E13 and E17. Before stress exposure control dams and dams that were appointed to the stress group showed the same gain in weight from E0 to E13 (see S 7 for more details). From beginning to end of the maternal stress paradigm (E13-E17), stressed dams showed reduced weight gain, when compared to control animals. The average litter size did not differ between groups.

S 7 Table Goup sizes, weight [g] of dams from embryonic day 0 to embryonic day 17 and weight gain (E13-E17, [%]), as well as litter size [n].

Condition	n	weight E0	weight E13	weight E17*	weight gain [%]*	n˚ pups
C	14	20.14 ± 0.20	30.82 ± 0.47	38.18 ± 0.85	19.4±0.01	7.36 ± 0.46
PS	22	20.29 ± 0.20	30.82 ± 0.32	35.75 ± 0.42	13.6±0.01	7.64 ± 0.31

The displayed values represent group means ± standard errors. Statistical significance ($p \leq 0.050$) as tested using the Kruskal-Wallis test is indicated by an asterisk. PS=prenatal stress, C=control, E=embryonic day

The efficacy of the PS paradigm can be seen by looking at the dam weight. A significant reduction in weight gain during the last stage of pregnancy was observed in dams, subjected to restraint stress in comparison to control dams, while both groups produced on average litters of equal size. Repeated, acute, restraint stress has been widely associated with a decrease in body weight in rodents (Rybkin *et al.*, 1997; Harris *et al.*, 2002, 2006; Jeong, Lee and Kang, 2013), suggesting that the dams subjected to the stress paradigm were indeed experiencing stress.

Gene expression and H3K4 tri-methylation

S 8 Table Top 10 differentially expressed genes (A) and differentially enriched loci (B) with regards to H3k4me3 histone modification, ranked by pvalue.

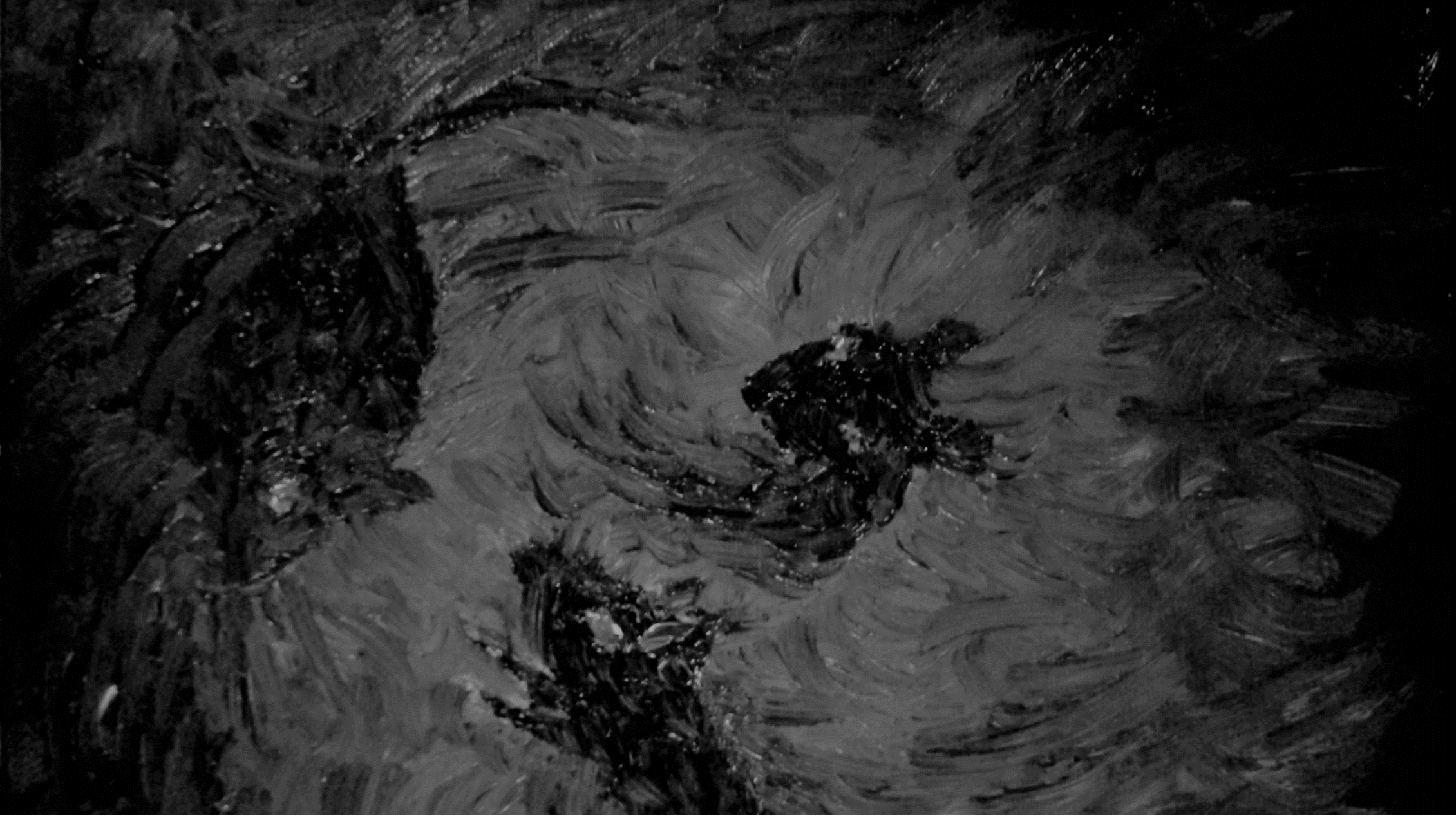
Symbol	Chr	Ensembl ID	Biotype	Start	End	Strand	Bm	lg2FC	lfcSE	stat	p-value	padj.
A)	*5-Htt*$^{+/+}$											
SUS - control												
Gm44798	8	ENSMUSG00000109139	TEC	9618153	9620562	-	7	-0.59	0.12	-4.75	2.00E-06	7.53E-02
Gm44801	8	ENSMUSG00000109529	TEC	9603823	9605882	-	4	-0.47	0.10	-4.66	3.20E-06	7.53E-02
Gm44799	8	ENSMUSG00000109095	TEC	9658248	9659449	-	5	-0.52	0.12	-4.51	6.35E-06	7.75E-02
4930435N07 Rik	8	ENSMUSG00000109551	TEC	9459092	9461218	+	3	-0.41	0.09	-4.51	6.58E-06	7.75E-02
Gm44797	8	ENSMUSG00000109359	TEC	9595109	9596945	-	4	-0.26	0.07	-3.64	2.77E-04	1.00E+00
Gm44800	8	ENSMUSG00000109418	TEC	9598799	9600653	-	4	-0.25	0.07	-3.46	5.40E-04	1.00E+00
Gm22396	1	ENSMUSG00000093782	miRNA	82839446	82839498	+	6	0.49	0.14	3.42	6.36E-04	1.00E+00
Gm12523	3	ENSMUSG00000084863	antisense	108283898	108284843	-	44	0.53	0.17	3.22	1.28E-03	1.00E+00
Gm24157	11	ENSMUSG00000093006	miRNA	52099043	52099153	-	127	0.51	0.16	3.15	1.62E-03	1.00E+00
Gm3448	17	ENSMUSG00000079710	protein_coding	14964189	15041537	-	2484	0.31	0.10	3.14	1.68E-03	1.00E+00
RES - control												
Gm45010	7	ENSMUSG00000109015	TEC	28167275	28169627	-	18	-0.25	0.07	-3.50	4.57E-04	1.00E+00
Plb1	5	ENSMUSG00000029134	protein_coding	32232708	32366520	+	38	0.25	0.07	3.41	6.48E-04	1.00E+00
Fabp7	10	ENSMUSG00000019874	protein_coding	57784881	57788450	+	201	-0.21	0.07	-3.05	2.25E-03	1.00E+00
Scd4	19	ENSMUSG00000050195	protein_coding	44333092	44346743	+	273	-0.21	0.07	-3.04	2.37E-03	1.00E+00
Gm43298	5	ENSMUSG00000107047	lincRNA	150863963	150882642	-	67	0.22	0.07	3.02	2.52E-03	1.00E+00
Gm11638	11	ENSMUSG00000086487	processed_transcript	105363252	105390642	+	63	0.22	0.07	3.01	2.58E-03	1.00E+00
4833418N02 Rik	17	ENSMUSG00000085287	lincRNA	87274886	87282814	-	47	0.22	0.07	2.98	2.86E-03	1.00E+00
Cdkn1a	17	ENSMUSG00000023067	protein_coding	29090979	29100722	+	92	-0.22	0.07	-2.96	3.03E-03	1.00E+00
Gm37435	1	ENSMUSG00000102676	TEC	40582895	40586841	-	46	0.22	0.07	2.96	3.06E-03	1.00E+00
Gm16577	10	ENSMUSG00000090070	antisense	11212280	11212979	-	30	0.21	0.07	2.92	3.51E-03	1.00E+00

	***5-Htt*$^{+/-}$**																
SUS - control																	
Sgk1	10						ENSMUSG00000019970	protein_coding	21882184	21999903	+	1347	-0.43	0.07	-6.45	1.09E-10	2.15E-06
Mt1	8						ENSMUSG00000031765	protein_coding	94179089	94180325	+	1544	-0.39	0.07	-5.77	8.08E-09	7.98E-05
Pim3	15						ENSMUSG00000035828	protein_coding	88862186	88865718	+	378	-0.30	0.06	-4.84	1.31E-06	7.19E-03
Sdc4	2						ENSMUSG00000017009	protein_coding	164424247	164443887	-	1145	-0.29	0.06	-4.77	1.80E-06	7.19E-03
Lfng	5						ENSMUSG00000029570	protein_coding	140607320	140615545	+	202	-0.33	0.07	-4.76	1.96E-06	7.19E-03
Ppp1r3g	13						ENSMUSG00000050423	protein_coding	35958839	35970388	+	93	-0.35	0.07	-4.74	2.19E-06	7.19E-03
Nfkbia	12						ENSMUSG00000021025	protein_coding	55489411	55492647	-	230	-0.32	0.07	-4.62	3.75E-06	1.06E-02
Mfsd2a	4						ENSMUSG00000028655	protein_coding	122946850	122961188	-	332	-0.29	0.06	-4.59	4.50E-06	1.11E-02
RP23-185B22.1	8						ENSMUSG00000109992	lincRNA	60719291	60725806	+	13	0.29	0.06	4.43	9.32E-06	NA
Slc2a1	4						ENSMUSG00000028645	protein_coding	119108711	119137983	+	1246	-0.31	0.07	-4.39	1.12E-05	2.30E-02
RES - control																	
Sgk1	10						ENSMUSG00000019970	protein_coding	21882184	21999903	+	1347	-0.38	0.07	-5.78	7.66E-09	1.37E-04
Nfkbia	12						ENSMUSG00000021025.7	protein_coding	55489411	55492647	-	230	-0.36	0.07	-5.30	1.16E-07	1.04E-03
Pim3	15						ENSMUSG00000035828	protein_coding	88862186	88865718	+	378	-0.31	0.06	-5.01	5.35E-07	3.19E-03
Serpine1	5						ENSMUSG00000037411	protein_coding	137061504	137072268	-	9	-0.29	0.06	-4.75	2.03E-06	NA
Mag	7						ENSMUSG00000036634	protein_coding	30899176	30914873	-	1482	-0.33	0.07	-4.73	2.27E-06	6.46E-03
Rtkn	6						ENSMUSG00000034930	protein_coding	83135463	83152579	+	653	-0.23	0.05	-4.67	3.02E-06	6.46E-03
Sgsh	11						ENSMUSG00000005043	protein_coding	119343425	119355536	-	119	-0.30	0.06	-4.64	3.56E-06	6.46E-03
Mt1	8						ENSMUSG00000031765	protein_coding	94179089	94180325	+	1544	-0.31	0.07	-4.60	4.30E-06	6.46E-03
Gm12355	11						ENSMUSG00000078134	protein_coding	98624351	98625661	-	1056	0.22	0.05	4.59	4.50E-06	6.46E-03
Slc2a1	4						ENSMUSG00000028645	protein_coding	119108711	119137983	+	1246	-0.32	0.07	-4.58	4.69E-06	6.46E-03
Peak	**Chr**	**start**	**end**	**width**	**Location**	**Distance**	**related Feature**	**Ensembl ID**	**Biotype**	**Start**	**End**	**Strand**	**Bm**	**lg2FC**	**lfcSE**	**stat**	**p-value**	**padj.**
B)	***5-Htt*$^{+/+}$**																
SUS - control																	

10335	16	42255414	42256268	855	downstream	48055	*mmu-mir-6540*	ENSMUSG00000098300	miRNA	42303362	42303469	-	88	0.59	0.15	3.86	1.14E-04	1.00E+00
22723	7	19075570	19077250	1681	overlapStart	-679	*Dmwd*	ENSMUSG00000030410	protein_coding	19076249	19082776	+	1249	0.21	0.06	3.60	3.16E-04	1.00E+00
1488	1	177442310	177443616	1307	overlapStart	960	*Gm26801*	ENSMUSG00000097892	lincRNA	177429047	177443270	-	1413	0.28	0.08	3.44	5.74E-04	1.00E+00
11673	17	56039939	56040820	882	overlapStart	-477	*Chaf1a*	ENSMUSG00000002835	protein_coding	56040416	56068026	+	517	-0.22	0.06	-3.35	8.11E-04	1.00E+00
10828	17	6476249	6477572	1324	overlapStart	853	*Tmem181b-ps*	ENSMUSG00000079733	protein_coding	6451888	6477102	-	661	0.35	0.10	3.34	8.29E-04	1.00E+00
25529	8	107435922	107437082	1161	overlapStart	-476	*Wwp2*	ENSMUSG00000031930	protein_coding	107436398	107558594	+	1246	0.23	0.07	3.31	9.49E-04	1.00E+00
9143	15	73644495	73646113	1619	overlapStart	1267	*Slc45a4*	ENSMUSG00000079020	protein_coding	73577424	73645762	-	1196	0.23	0.07	3.30	9.81E-04	1.00E+00
5630	12	56636543	56637475	933	upstream	-23259	*Nkx2-9*	ENSMUSG00000058669	protein_coding	56611389	56613284	-	60	-0.54	0.17	-3.28	1.06E-03	1.00E+00
24285	7	140821271	140822333	1063	overlapStart	907	*Zfp941*	ENSMUSG00000060314	protein_coding	140807449	140822178	-	282	-0.31	0.10	-3.20	1.40E-03	1.00E+00
12196	18	20187979	20188745	767	upstream	-59361	*Dsg1c*	ENSMUSG00000034774	protein_coding	20247340	20285031	+	64	0.56	0.18	3.19	1.42E-03	1.00E+00
RES - control																		
26527	9	58822288	58824077	1790	overlapStart	-1224	*Hcn4*	ENSMUSG00000032338	protein_coding	58823512	58860955	+	951	-0.32	0.06	-5.15	2.64E-07	5.45E-03
11854	17	72603065	72605026	1962	overlapStart	644	*Alk*	ENSMUSG00000055471	protein_coding	71869442	72603709	-	408	-0.39	0.08	-4.75	2.01E-06	2.07E-02
6156	12	106310512	106311356	845	upstream	-16856	*Gm17032*	ENSMUSG00000091280	lincRNA	106327368	106330821	+	97	-0.72	0.16	-4.45	8.45E-06	5.73E-02
5239	12	8301522	8302393	872	overlapStart	432	*Gdf7*	ENSMUSG00000037660	protein_coding	8297934	8301954	-	169	-0.57	0.13	-4.35	1.33E-05	5.73E-02
10773	16	97610357	97611405	1049	overlapStart	838	*Tmprss2*	ENSMUSG00000000385	protein_coding	97564684	97611195	-	83	-0.88	0.20	-4.35	1.39E-05	5.73E-02
3696	11	54859858	54861228	1371	overlapStart	1058	*Lyrm7*	ENSMUSG00000020268	protein_coding	54826866	54860916	-	658	0.23	0.05	4.30	1.69E-05	5.81E-02
16564	3	82875855	82876796	942	overlapStart	628	*Rbm46*	ENSMUSG00000033882	protein_coding	82837228	82876483	-	142	-0.66	0.16	-4.22	2.41E-05	6.39E-02
9696	15	99096726	99097450	725	inside	3556	*Dnajc22*	ENSMUSG00000038009	protein_coding	99093170	99104737	+	97	-0.78	0.18	-4.22	2.48E-05	6.39E-02
20872	5	140975479	140976052	574	inside	25117	*Card11*	ENSMUSG00000036526	protein_coding	140872999	141000596	-	10	2.82	0.69	4.07	4.73E-05	NA
25873	8	125910126	125911500	1375	overlapStart	-324	*BC021891*	ENSMUSG00000031853	protein_coding	125910450	125947439	+	705	-0.27	0.07	-4.02	5.83E-05	9.87E-02
		***5-Htt*$^{+/-}$**																
SUS - control																		
25839	8	123653434	123655061	1628	overlapStart	-495	*Rhou*	ENSMUSG00000039960	protein_coding	123653929	123663884	+	1169	-0.28	0.06	-4.56	5.16E-06	1.42E-01
276	1	42696319	42698075	1757	overlapStart	-827	*Pou3f3*	ENSMUSG00000045515	protein_coding	42697146	42700207	+	1413	-0.25	0.06	-4.42	1.01E-05	1.42E-01
6082	12	100519641	100521258	1618	overlapStart	1181	*Ttc7b*	ENSMUSG00000033530	protein_coding	100300771	100520822	-	1207	-0.23	0.06	-4.12	3.81E-05	3.60E-01
13377	19	24030073	24031493	1421	overlapStart	946	*Fam189a2*	ENSMUSG00000071604	protein_coding	23972751	24031019	-	998	-0.29	0.07	-3.98	7.04E-05	4.48E-01

9143	15	73644495	73646113	1619	overlapStart	1267	*Slc45a4*	ENSMUSG00000079020	protein_coding	73577424	73645762	-	1196	-0.28	0.07	-3.94	8.05E-05	4.48E-01
10736	16	94568873	94569424	552	upstream	-1137	*Dyrk1a*	ENSMUSG00000022897	protein_coding	94570010	94695517	+	425	-0.28	0.07	-3.90	9.49E-05	4.48E-01
5214	12	4475600	4477595	1996	overlapStart	1582	*Ncoa1*	ENSMUSG00000020647	protein_coding	4247363	4477182	-	2260	-0.23	0.06	-3.86	1.12E-04	4.52E-01
27010	9	102834478	102836041	1564	overlapStart	-439	*Ryk*	ENSMUSG00000032547	protein_coding	102834917	102908305	+	1478	-0.26	0.07	-3.79	1.52E-04	5.39E-01
16638	3	88030796	88031775	980	upstream	-3213	*Hapln2*	ENSMUSG00000004894	protein_coding	88021750	88027583	-	97	-0.65	0.17	-3.75	1.74E-04	5.47E-01
20647	5	129715120	129716196	1077	overlapStart	-377	*Mrps17*	ENSMUSG00000034211	protein_coding	129715497	129718875	+	710	0.20	0.06	3.69	2.27E-04	6.43E-01
RES - control																		
6758	13	42956466	42957701	1236	upstream	-42045	*Gm15809*	ENSMUSG00000090135	sense_intronic	42998511	43006067	+	89	0.77	0.18	4.26	2.06E-05	3.23E-01
276	1	42696319	42698075	1757	overlapStart	-827	*Pou3f3*	ENSMUSG00000045515	protein_coding	42697146	42700207	+	1413	-0.24	0.06	-4.13	3.61E-05	3.23E-01
26034	9	21183956	21184657	702	inside	18242	*Pde4a*	ENSMUSG00000032177	protein_coding	21165714	21213248	+	127	-0.57	0.14	-4.09	4.26E-05	3.23E-01
12294	18	33999093	33999993	901	inside	8113	*Epb4.1l4a*	ENSMUSG00000024376	protein_coding	33796327	34007206	-	45	0.81	0.20	4.07	4.76E-05	3.23E-01
13576	19	41085868	41086414	547	upstream	-8755	*Opalin*	ENSMUSG00000050121	protein_coding	41062474	41077113	-	35	-0.96	0.24	-4.01	6.08E-05	3.23E-01
6456	13	21171726	21172146	421	upstream	-7719	*Trim27*	ENSMUSG00000021326	protein_coding	21179445	21194724	+	206	0.46	0.12	3.98	6.84E-05	3.23E-01
10196	16	31934927	31935754	828	inside	1076	*Pigz*	ENSMUSG00000045625	protein_coding	31933851	31946046	+	177	-0.52	0.13	-3.90	9.62E-05	3.89E-01
15003	2	104256546	104256920	375	overlapStart	-126	*Gm13883*	ENSMUSG00000087473	antisense	104256672	104275757	+	15	-1.39	0.37	-3.80	1.43E-04	5.08E-01
8098	14	55014469	55015204	736	upstream	-985	*Ngdn*	ENSMUSG00000022204	protein_coding	55015454	55024135	+	222	-0.37	0.10	-3.75	1.78E-04	5.37E-01
5316	12	17556819	17557365	547	downstream	10673	*Gm22748*	ENSMUSG00000064427	snoRNA	17546146	17546281	+	45	-0.90	0.24	-3.73	1.95E-04	5.37E-01

Genes and associated histone mark H3K4me3, affected by increased susceptibility (SUS) or resilience (RES), dependent on the serotonin transporter (*5-Htt*) genotype. Chr=chromosome, Bm=base mean, lg2FC= log2 fold change, lfcSE=log2 fold change standard error, stat=Wald-statistic, lincRNA=long intergenic non-coding RNA, lncRNA=long non-coding RNA, padj=adjusted p-value, TEC=to be experimentally confirmed. Data based on sequencing counts of total RNA and MBD enriched loci (group size n = 6-8).



Differential anxiety-related behaviours and brain activation in Tph2-deficient female mice exposed to adverse early environment

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Abstract

Anxiety disorders represent one of the most prevalent mental disorders in today's society and early adversity has been identified as major contributor to anxiety-related pathologies. Serotonin (5-hydroxytryptamine, 5-HT) was found to be implicated in mediating the effects of early-life events on anxiety-like behaviours. In order to further elucidate the interaction of genetic predisposition and adversity in early, developmental stages on anxiety-related behaviours, the current study employed tryptophan hydroxylase 2 (*Tph2*)–deficient female mice, as a model for lifelong brain 5-HT synthesis deficiency. Offspring of this line were exposed to neonatal maternal separation (MS) and tested, in adulthood, in the open-field (OF) or the dark-light box (DLB). Subsequently, neural activity was assessed, using c-Fos immunohistochemistry. In the DLB, MS rescued the observed decrease in activity in the light compartment of homozygous *Tph2*–deficient mice. In the OF, MS increased escape-related behaviours in *Tph2*-deficient offspring. On the neural level, both behavioural tests evoked a distinct activation pattern, as shown by c-Fos immunohistochemistry. Exposure to the DLB resulted in *Tph2*-dependent activation of paraventricular nucleus and basolateral amygdala, while OF exposure lead to a specific activation in lateral amygdala of maternally separated animals. Taken together, our findings suggest that MS promotes active responses to aversive stimuli, dependent on the availability of brain 5-HT. These effects might be mediated by the distinct activation of anxiety-relevant brain regions, due to the behavioural testing.

Introduction

Anxiety disorders, such as agoraphobia and generalised anxiety disorder (GAD) are a major social and economic burden in today's society (Kessler and Greenberg, 2012). They comprise a cluster of disorders, characterised by dysregulated physiological and psychological functions, involving e.g. abnormal autonomic and neuroendocrine activation, leading to (hyper-) increased arousal (American Psychiatric Association, 2013). Anxiety can be described as a response to potential danger and often emanates from more obscure, unpredictable threats when compared to fear, which involves more explicit, direct threat (Davis *et al.*, 2010). As such, anxiety supports successful adaption to a changing environment. Maladaptive forms of anxiety as in e.g. misrepresentation of anticipated consequences or reacting to non-fearful stimuli, can lead to severe impairments in everyday life (Steimer, 2011). Fear is a more bodily response to acute threat. It is prompted by acute threat and elicits active defensive responses (Davis *et al.*, 2010). Panic attacks are ranged as a particular type of fear disorder with symptoms that occur idiopathic and overlap highly with, but exaggerate, fear responses, as observed under normal conditions (American Psychiatric Association, 2013). In animal studies, behavioural inhibition is one of the most comprehensible measures to evaluate anxiety (Steimer, 2011), while panic behaviour is evaluated by measuring physiological parameters and escape-behaviour (Paul *et al.*, 2014). Both behavioural profiles, i.e. anxiety- and fear-related behaviours, can be investigated using aversion related behavioural tests, such as the open-field (OF) and dark-light box test (DLB), where increased behavioural inhibition (Holmes *et al.*, 2003) or exaggerated fear can be observed (Waider *et al.*, 2017).

One major risk factor for the development of an anxiety disorder is the exposure to adverse conditions throughout early-life (Heim and Nemeroff, 2001; Weinstock, 2008; van den Bergh *et al.*, 2017). Spadework in the field of early-life adversity showed that adverse experiences throughout development have the capacity to affect the stress response later in life, mostly by reprogramming the reactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Thompson, 1957; Levine, 1967). Several studies, in multiple species, identified a reciprocal regulation of serotonin (5-hydroxytryptamine; 5-HT) system functioning and stress exposure as crucial mechanism of early adversity (Booij *et al.*, 2015). While alterations in the 5-HT system were observed to alter the effects of early adversity on later behavioural phenotypes (van den Hove *et al.*, 2011; Sachs *et al.*, 2015; Wong *et al.*, 2015), early adversity was able to alter the expression of relevant components of the 5-HT system (Gardner et al. 2009 a; Gardner et al. 2009 b; Hiroi et al. 2016; Holloway et al. 2013; van den Hove et al. 2011; Wong et al. 2015). Consequently, an interaction between early-life stress and functioning of the 5-HT system exerts an effect on the activity of limbic brain structures and HPA axis and, through such, on the reactivity to diverse anxiety-related tests. Thereby, the 5-HT system provides a modulatory interface, allowing or restricting the effects of early adversity.

The current study investigated the interaction of early, adverse life experience and genetic 5-HT deficiency in female mice, using tryptophan hydroxylase 2 null mutant (*Tph2*$^{-/-}$), heterozygous (*Tph2*$^{+/-}$) and wildtype (*Tph2*$^{+/+}$) mice that were or were not exposed to maternal separation (MS). During adulthood, two distinct anxiety tests, the dark-light box (DLB) and open-field (OF) test were used as read out for anxiety-like behaviours. Next to the behavioural investigation, c-Fos immunohistochemistry was used to analyse neuronal activity in several brain regions that have been

identified as key anxiety-related, regulatory regions under the control of the 5-HT-system (Paul and Lowry, 2013), including the basolateral (BL), lateral (La) and central (Ce) nucleus of the amygdala, the paraventricular nucleus (PVN) and the ventrolateral (VLPAG) and dorsolateral (DLPAG) periaqueductal grey.

Experimental procedures

Animals and procedures

All experiments were performed in accordance with the European Parliament and Council Directive (2010/63/EU) and were approved by local authorities (Würzburg: 55.2-2531.01- 57/12). All efforts were made to minimize animal numbers and suffering of the animals. All experimental animals were bred, kept and tested in the centre for experimental molecular medicine (ZEMM) in Würzburg. The study was performed in two batches at a difference of one month, with the first cohort of animals used for OF testing, while the second cohort was used for DLB testing. Each test cohort, included, had a complementary group of naïve animals that was not exposed to the test.

The parental generation of male and female mice were housed in groups of 2-7 under 14 h/10 h light-dark cycle with lights on at 7AM and lights off at 9PM, at 21±1°C, with 45-55% humidity. Standard rodent chow and water were available *ad libitum*. For breeding, two female, and one male *Tph2*$^{+/-}$ mice, fully backcrossed onto C57BL/6N genetic background and approximately 3 months of age were put together. Females were checked for the occurrence of vaginal plugs twice a day. After 5 days all mating pairs were separated and all females that were tested plug positive at least once were housed alone. From day 14 after the separation of mating pairs the cages were checked for pups. The day of birth was declared postnatal day (P) 0.

From P2 until P15, about half of the dams and respective litters were subjected to a maternal separation paradigm (MS) adapted from Veenema and colleagues (Veenema *et al.*, 2006), while the other half were normally reared and served as controls. The groups were appointed randomly and number of pups did not differ significantly between groups, neither within, nor between cohorts. In detail, the pups together with the respective nesting material were taken out of the mother's cage and brought into an adjacent room, where they were kept under heating lamps at approximately 29°C and humidity levels above 60% for 3 h. The separation time point was randomly assigned in the light interval. Control dams were left undisturbed in their home cages except for P5, P10 and P15 where litters and mothers were weighted and P5, P12 and P19 when the cages were changed. On P25±3, pups were weaned and female offspring were housed in groups of 6±2 according to genotype and condition (MS or control) and were allowed to grow up undisturbed except for weekly cage changes, weighing (S 2) and estrus determination on around P41 and P55 (S 1) as described in detail in (McLean *et al.*, 2012). Behavioural testing was performed in female offspring at the age of two months during the post-ovulatory phase (met-estrus/di-estrus) to avoid hormonal variation.

Animals were tested either in the DLB (n = 45, n = 6-9 per group) or in the OF (n = 45, n = 4-9 per group) test. For each test, animals were randomly assigned to either the test or naïve group. The order, in which animals were tested, was randomised over genotype and MS group. For all tests, mice were

tracked using infrared light from below the respective apparatus. Trials were recorded from above, using an infrared-sensitive camera. Later on, behavioural analysis was performed using VideoMot2 tracking software (TSE Systems, Bad Homburg, Germany). In-between trials the respective apparatus was cleaned with Terralin liquid (Schülke, Norderstedt, Germany). The animals were tested during the light phase (8AM - 1PM).

Dark-light box test

The DLB test represents an ambiguous environment and is based on the conflicting urge to explore unknown territory and to avoid brightly lit, open areas (Crawley and Goodwin, 1980; Onaivi and Martin, 1989). The DLB is an arena made from opaque white acrylic glass with an edge length of 50 cm and wall height of 40 cm. This arena is split into a light compartment (34 cm x 50 cm) with 100 lux in the centre and 70 lux in the corners and a dark compartment (16 cm x 50 cm) with no light exposure. The two compartments are connected through an exit hole and the animal can move freely between compartments (Waider *et al.*, 2017). Each tested animal was placed into the dark compartment, facing the front right corner, and was allowed to explore the arena freely for 10 min. The total distance covered in the whole arena as well as time and visits in the light compartment, and the latency to enter the light compartment were determined. Furthermore, rearing, jumping, and an initial delay to locomotion were hand scored from the recorded video files by an experimenter blinded for genotype and condition. 6-9 animals were tested per group. Rearing and jumping were analysed separately in the light and in the dark and are reported relative to the time spent in the respective compartment.

Open-field test

The OF is an open arena made from opaque acrylic glass with an edge length of 50 cm and a wall height of 40 cm. The arena was brightly illuminated with 90 lux in the centre and 50 lux in the corners (Waider *et al.*, 2017). Each tested animal was placed into the arena facing the front right corner and was allowed to explore the arena freely for 10 min. The time in the centre, the latency to enter the centre, visits in the centre, time per visit in the centre and distance moved in the centre and over the whole arena were determined (Hall, 1934; Seibenhener and Wooten, 2015). Rearing, jumping and an initial delay to locomotion were hand scored from the recorded video files, by an experimenter blinded for genotype and condition. 4-9 animals were tested per group.

Tissue processing

Two hours following the testing, behaviourally tested offspring were sacrificed and perfused with 4% PFA (dissolved in PBS, pH7.5). Behaviourally naïve animals were sacrificed on the same days. Brains were taken and post-fixed in 4% PFA for 48 h at 4°C, immersed in 10% and 20% sucrose and finally frozen at -80°C in isopentane. Subsequently, 30 µm thick sections were cut from Bregma -0.22 mm to -5.80 mm using a Cryostat (Leica Biosystems, Nussloch, Germany) and mounted on Histobond+ slides (Marienfeld Superior, Lauda-Königshofen, Germany) in the order of 6 series with an interval of 180 µm between adjacent sections.

Anti c-Fos immunohistochemistry

For the investigation of neural activation, c-Fos immunohistochemistry was performed. c-Fos is an immediate early gene and c-Fos expression is notably increased following neural activation (Hale *et al.*, 2006; Hale, Hay-schmidt, Mikkelsen, Poulsen and Lowry, 2008; Hale, Hay-schmidt, Mikkelsen, Poulsen, Adriaan, *et al.*, 2008). The frozen sections were air-dried for 30 min and following several rinses in 1x PBS, subjected to antigen retrieval in sodium citrate buffer (pH 6.0 at 80°C for 20 min). Antigen retrieval was followed by inactivation of peroxidases with 0.6% hydrogen peroxide in 1x PBS for 30 min at room temperature (RT). Subsequently, the slides were incubated with blocking solution (5% normal horse serum (NHS), 2% bovine serum albumin (BSA), 0.25% Triton X-100 in 1x PBS) for 2 h, at RT, and incubated for 48 h at 4°C with an antibody against c-Fos (1:2000, sc-52 Lot. F1715, Santa Cruz, Dallas, Texas, USA). Every step until the blocking, during the first day of the protocol, was followed by a 3x 5 min washing step in 1x PBS at RT. Following primary antibody incubation, slides were washed three times for 10 min in 1x PBS at RT and incubated for 50 min at RT with 300 µl ImmPRESS (peroxidase) polymer anti-rabbit IgG solution (Cat. No. MP-7401, Vector Laboratories). Following a 3x 10 min wash with 1x PBS at RT, 300 µl of the Vector SG Peroxidase Substrate (Cat. No. SK-4700, Vector Laboratories), prepared according to kit instructions, were added for 5-15 min. Subsequently, slides were washed 5x 7 min in 1x PBS and 2x 5 min in tap water, followed by Nuclear Fast Red (Vector H-3403) counterstaining. Finally, slides were dehydrated and cover-slipped using Vitro Clud (NOVOGLAS, Langenbrinck, Germany).

Stereological analysis

To analyse the number of c-Fos immunoreactive cells (c-Fos$^+$) in several amygdaloid nuclei (BL, La, Ce), PAG and PVN, we used a computer microscopy system with the Stereo Investigator software (MBF Biosciences, Williston, VT, USA). With this system we counted c-Fos$^+$ cells using design-based stereology. Briefly, the BL, La and Ce (from Bregma level -0.70 mm to-2.40 mm), PAG (from Bregma level -4.16 mm to -4.96 mm) and PVN (from Bregma level -0.46 mm to -1.06 mm) were delineated at a 4x magnification on live microscopic images, displayed on a monitor. Subsequently, cells were counted at a 20x magnification, using the optical fractionator workflow. To correct for the decrease in volume observed in several of the investigated regions of *Tph2*$^{-/-}$ animals (S 5), subsequent analyses were performed using the density (ρA) of c-Fos$^+$ cells per region.

Statistics

Statistics on behavioural and immunohistological data were performed using IBM SPSS Statistics (IBM Deutschland GmbH, Ehningen, Germany). Data were investigated for normal distribution, using Shapiro-Wilks test and visually by examining the histograms. If the assumption of normal distribution was not met, data were either analysed using the non-parametrical Kruskal-Wallis test for multi-factorial analysis (*Tph2* genotype and MS) followed by Mann-Whitney-U, or transformed, using either log10 or square root transformation, and tested using multifactorial ANOVA. To transform data, we started with the log10 transformation, followed by test for normal distribution. If log10 transformation was not successful at establishing normality, square root transformation was performed. For all data, subsequently tested using multifactorial ANOVA (*Tph2* genotype* MS*test condition) followed by

Bonferroni-corrected t-tests, either log10 or square root transformation were able to establish normality. Correlations were calculated using Spearman's correlation coefficient. P-values of $p \leq 0.050$ were considered significant. For comparison of categorical results Chi-Square test was performed.

Results

Maternal separation rescues anxiety-related behaviour in tryptophan hydroxylase 2-deficient mice in the dark-light box

In the DLB test, animals of all groups showed a comparable latency to enter the light compartment (Figure 1A) and in the first 5-minute testing interval, no differences in the time spent in the light compartment was observed between groups (Figure 1B, left panel). The time spent in light during the first visit of the light compartment differed significantly between groups ($\chi^2(5) = 15.7$, $p = 0.008$;). *Tph2*$^{-/-}$ ($U = 2.0$, $p = 0.003$) and *Tph2*$^{+/-}$ ($U = 8.5$, $p = 0.040$) animals that experienced MS stayed longer than their *Tph2*$^{+/+}$ counterparts in the bright compartment. In control offspring, *Tph2*$^{-/-}$ animals spent more time during the first visit when compared to *Tph2*$^{+/-}$ control offspring ($U = 10.0$, $p = 0.045$; see S 3 A) In the second 5 min testing-interval, MS offspring spent more time in the light compartment than control offspring ($U = 145.0$, $p = 0.023$, Figure 1B, right panel). This effect also approached significance over the full 10 min testing interval ($U = 168.0$, $p = 0.082$, see S 3 C).

Experimental groups differed significantly for the total rearing frequency in the light compartment ($\chi^2(5) = 12.6$, $p = 0.027$; Figure 1 C, upper panel). *Tph2*$^{-/-}$ offspring of the control group reared with a lower frequency compared to *Tph2*$^{+/-}$ ($U = 8.0$, $p = 0.039$) and *Tph2*$^{+/+}$ ($U = 8.0$, $p = 0.039$) offspring of the control group and to *Tph2*$^{-/-}$ offspring of the MS group ($U = 2.0$, $p = 0.005$). Rearing frequencies in the dark did not differ between groups (Figure 1C, lower panel). Furthermore, the number of animals that jumped was dependent on the interaction between *Tph2* genotype and MS ($\chi(5) = 18.2$, $p = 0.003$) as well as the illumination of the area. (light compartment ($\chi(5) = 19.2$, $p = 0.002$), dark compartment ($\chi(5) = 4.4$, $p = 0.493$); Figure 1 D). Indeed, in the light compartment no effect was found in the control group ($\chi(2) = 3.8$, $p = 0.148$) although only 1/9 *Tph2*$^{+/+}$ (11,1%) and 1/8 *Tph2*$^{+/-}$ (12,5%) but 3/6 *Tph2*$^{-/-}$ (50%) jumped. MS was associated with an increased number of *Tph2*$^{-/-}$ offspring jumping (75%) in comparison to both *Tph2*$^{+/+}$ (0%) and *Tph2*$^{+/-}$ (0%) offspring ($\chi(2) = 14.4$, $p = 0.001$). No differences between groups were observed for the number of entries into the light, the total distance moved throughout the test or the latency to locomotion (S 3 B, D and E).

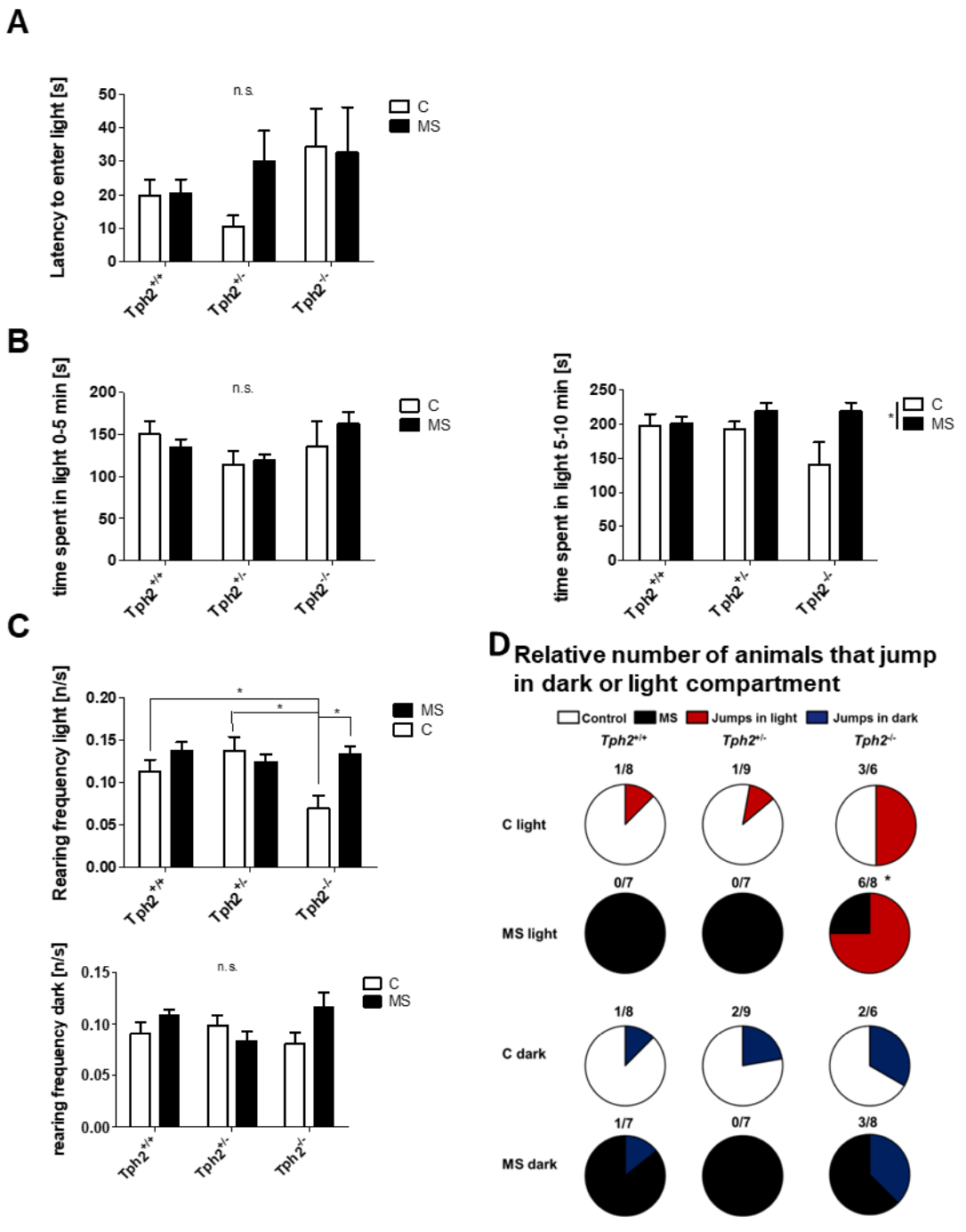

Figure 1 Anxiety-related behaviour in the dark-light box. (A) Latency to enter the light compartment [s] was not affected by either tryptophan hydroxylase 2 (*Tph2*) deficiency, or maternal separation (MS) or their interaction. (B) Time spent in the light compartment [s] during the first interval [0-5 min] and the second interval [5-10 min] of testing, where MS increased the time spent in the light during the second interval ($p = 0.023$) but not during the first interval. (C) Rearing frequency [n/s] in the dark and light compartment, with increased rearing following MS in light, dependent on the *Tph2* genotype ($p < 0.050$). Values represent group means ± standard errors (n = 6-9). *$p < 0.050$, (Post hoc: Mann-Whitney U). (D) Pie-charts of relative number of animals jumping per group in light (red) or dark (blue). *$p < 0.050$ (Chi-Square test for association).

C-Fos immunohistochemistry revealed a general activation of the La ($F(1,74) = 11.9$, $p = 0.001$) and Ce ($F(1,74) = 24.4$, $p < 0.001$) with mice, being exposed to the DLB, showing increased densities of c-Fos immunoreactive (c-Fos$^+$) cells, as compared to behaviourally naïve mice (Figure 2 C). In addition to this, we found a *Tph2* deficiency-dependent, distinct increase in c-Fos$^+$ cells in the BL ($F(2,74) =$

3.3, p = 0.043), namely with an observable increase in c-Fos^{+}, only in tested compared to non-tested *Tph2*$^{+/-}$ offspring (p < 0.001; Figure 2 C). In the PVN, density of c-Fos^{+} cells was affected by *Tph2* genotype (F(2,74) = 3.1, p = 0.026). Here, *Tph2*$^{-/-}$ animals seemed to show no increase in c-Fos immunoreactivity when compared to behaviourally naïve *Tph2*$^{-/-}$ animals, whereas as a significant difference was observed in *Tph2*$^{+/+}$ (p < 0.001) and *Tph2*$^{+/-}$ (p < 0.001) animals (Figure 2 F).

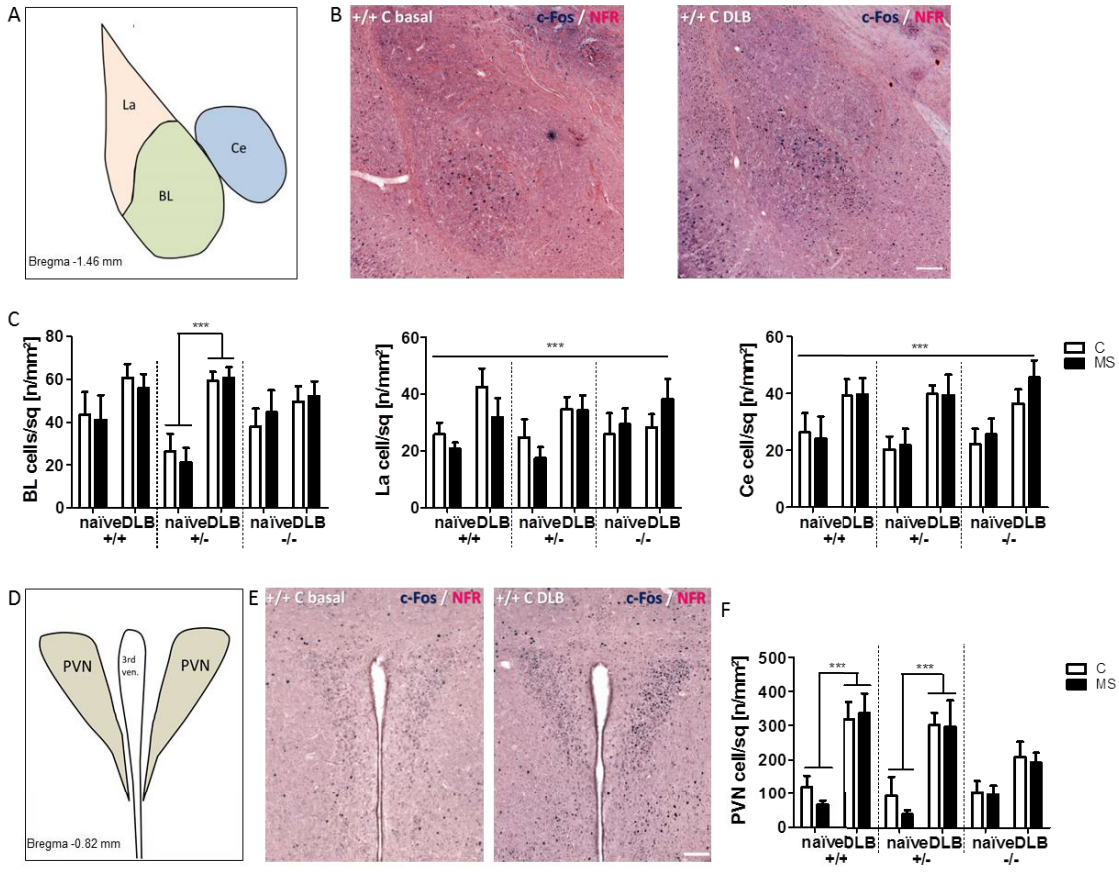

Figure 2 Neural activity in amygdala and paraventricular nucleus following dark-light box testing, as determined by c-Fos immunoreactivity. (A) Anatomical scheme, depicting the subnuclei of the amygdala. (B) Representative histological section of the c-Fos immunostaining at the amygdala at Bregma level -1.5 mm. (C) Behavioural testing in the dark-light box (DLB) leads to a general activation of the lateral (La) and central (Ce) nuclei of the amygdala and in the basolateral (BL) amygdala, dependent on the tryptophan hydroxylase 2 (*Tph2*) genotype (p < 0.050). (D) Anatomical scheme depicting the paraventricular nucleus (PVN). (E) Representative histological section of the c-Fos immunostaining at the PVN at Bregmal level -0.8 mm. (F) Behavioural testing in the DLB leads to a genotype-dependent activation (p < 0.050). Maternal separation (MS) had no effect on DLB-dependent neural activation. Bars represent group means ± standard error (n = 6-9). ***p < 0.001 (Post hoc: t-test, Bonferroni).

Moreover, the c-Fos immunoreactivity in the La correlated positively with the rearing frequency in the light (ρ = 0.318, p = 0.035) and in the dark (ρ = 0.474, p = 0.001) compartment, while the c-Fos immunoreactivity in both the BL (ρ = 0.389, p = 0.009) and Ce (ρ = 0.353, p = 0.019) only correlated positively with the rearing frequency in the dark compartment.

Maternal separation induces escape-related behaviour in tryptophan hydroxylase 2-deficient mice in the open-field

In the OF test, we observed a comparable distance moved in the centre and throughout the whole arena (Figure 3 A and B). Neither the latency to enter the centre, visits into the centre or time per visit in the centre (S 4 A-C), nor time spent in the centre (Figure 3 C) were affected by any of the investigated factors.

We observed a genotype-dependent decrease in rearing ($\chi^2(2) = 7.8$, $p = 0.019$) in *Tph2*$^{-/-}$ offspring of either condition, compared to *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ offspring ($p < 0.020$; Figure 3 D). Exposure to MS was associated with an increased number of animals, which frequently jumped in the course of the OF test, in comparison to their control counterparts ($\chi(1) = 5.1$, $p = 0.024$). The number of animals that jumped per group was affected by MS, in particular, in *Tph2*$^{+/-}$ animals with 0% jumping of the *Tph2*$^{+/-}$ control offspring and 44.4% jumping of the *Tph2*$^{+/-}$ MS offspring (Figure 3 E). Jumping only occurred after 5 min of testing and was positively associated with the visits in the centre ($\rho = 0.30$, $p = 0.045$) as well as the global distance covered ($\rho = 0.37$, $p = 0.012$) during this period. Furthermore, we found group-specific differences in the initial latency to locomotion ($\chi^2(5) = 14.4$, $p = 0.013$) as displayed in S 4 D. *Tph2*$^{-/-}$ animals that had experienced MS showed a longer latency to locomotion when compared to *Tph2*$^{+/+}$ ($U = 12.5$, $p = 0.040$) and *Tph2*$^{+/-}$ ($U = 9.0$, $p = 0.008$) offspring of the same group. *Tph2*$^{+/-}$ animals of the MS group, in contrast, showed the shortest latency, when compared to the two other genotypes ($p < 0.020$).

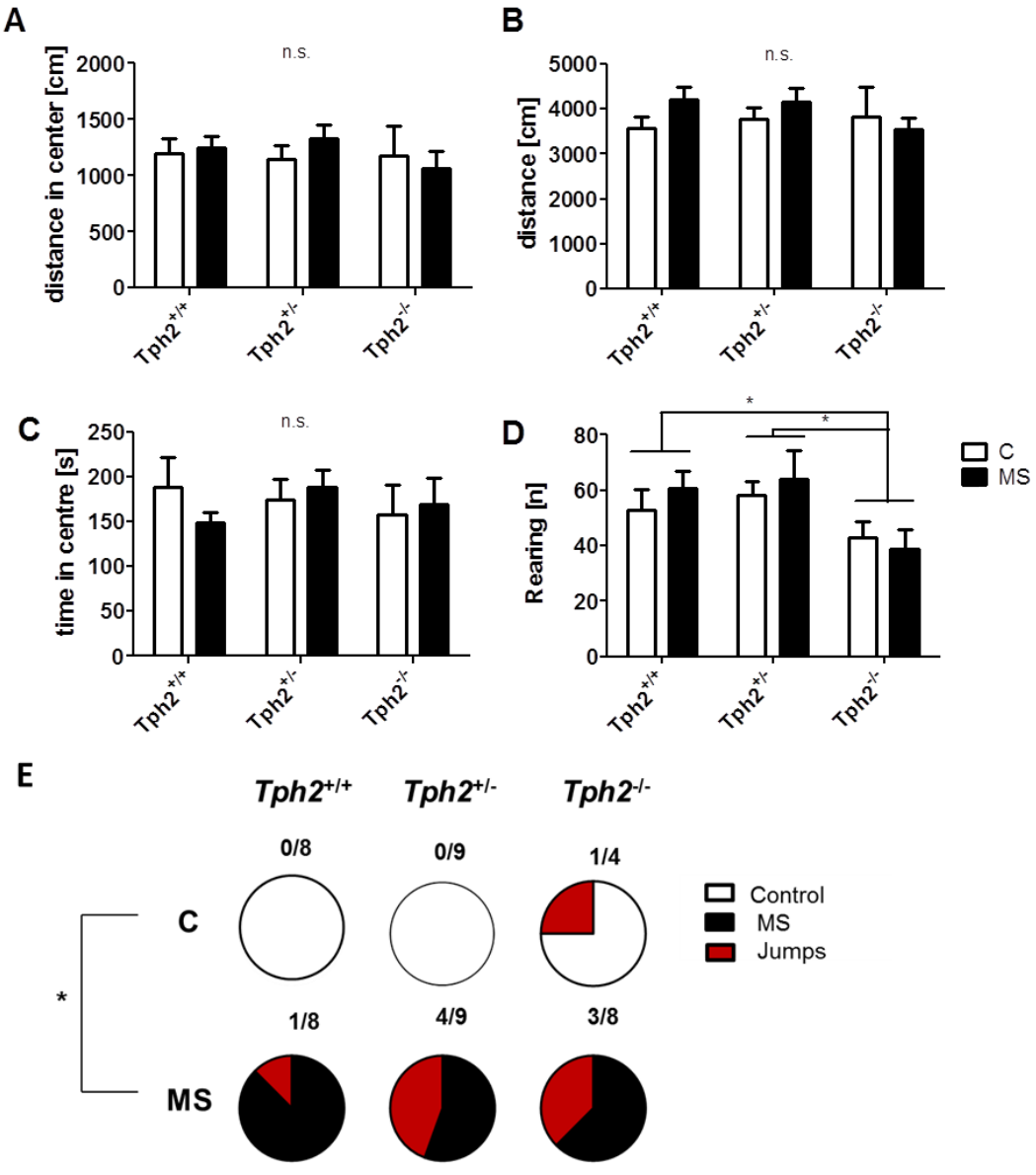

Figure 3 Anxiety-related behaviour in the open-field. Neither tryptophan hydroxylase 2 (*Tph2*) deficiency, nor maternal separation (MS) affected (A) the distance in the centre and (B) the total distance covered [cm] and (C) the time [s] in the centre. (D) Complete *Tph2* deficiency decreased the number of rearings ($p < 0.020$). *$p < 0.050$ (Post hoc: Mann-Whitney U). (E) MS increased the number of animals that jumped in the open-field (OF) during the 10 min test period [%] ($p = 0.030$). Bars represent group means ± standard errors ($n = 4$-9). Pie-charts of relative number of animals jumping per group. *$p < 0.050$ (Chi-Square test for association).

Analysis of c-Fos immunoreactivity revealed a general effect of OF testing on BL ($F(1,74) = 59.3$, $p < 0.001$), Ce ($F(1,74) = 17.3$ $p < 0.001$) and PVN ($F(1,74) = 70.8$, $p < 0.001$), with mice exposed to the OF showing higher c-Fos$^+$ cell densities compared to behaviourally naïve mice (Figure 4 C and F). Moreover, we observed a distinct activation of the La ($F(1, 74) = 5.9$, $p = 0.013$). In naïve MS offspring the c-Fos$^+$ cell density was lower when compared to naïve, non-stressed controls ($p = 0.008$), exposure to the OF increased the c-Fos$^+$ cell density in those animals ($p=0.001$), but did not alter the c-Fos$^+$ cell density in non-stressed, control animals.

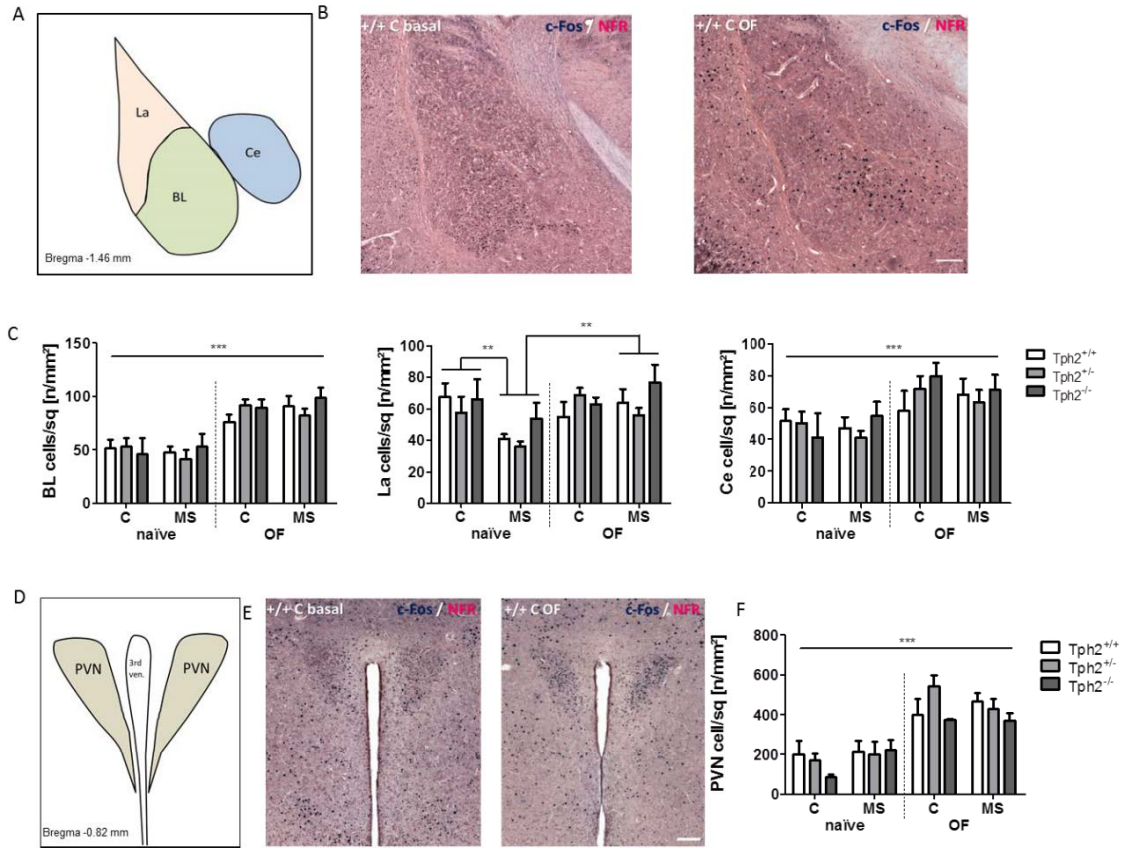

Figure 4 Neural activity in amygdala and paraventricular nucleus following open-field testing, as determined by c-Fos immunoreactivity. (A) Anatomical scheme depicting the subnuclei of the amygdala. (B) Representative histological section of the c-Fos immunostaining at the amygdala at Bregma level -1.5 mm. (C) Behavioural testing in the open-field (OF) leads to a general activation of the basolateral (BL) and central (Ce) nuclei of the amygdala and, in the lateral (La) amygdala, dependent on neonatal exposure to maternal separation (MS) ($p < 0.020$). (D) Anatomical scheme depicting the paraventricular nucleus (PVN). (E) Representative histological section of the c-Fos immunostaining at the PVN at Bregma level -0.8 mm. (F) Behavioural testing in the OF leads to a general activation ($p<0.001$), tryptophan hydroxylase 2 (*Tph2*) had no effect on OF-dependent neural activation. Bars represent group means ± standard error (n = 4-9). **$p < 0.010$ and ***$p < 0.001$ (Post hoc: t-test, Bonferroni).

In the VLPAG the relative c-Fos$^+$ cell density was observed to be altered dependent on a three-way interaction of genotype, early-life conditions and the OF test during adulthood ($F(2,74) = 3.8$, $p = 0.028$) (Figure 5C). Exposure to the OF test increased the density of c-Fos$^+$ cells only in *Tph2*$^{+/-}$ animals of the control ($p = 0.001$) and MS ($p = 0.001$) group and also the activation in *Tph2*$^{-/-}$ animals of the control group ($p = 0.002$) in comparison to activation in the complementary naïve animals, while in the DLPAG the 3-way interaction ($F(2, 74) = 3.8$ $p = 0.026$) did not show any significance in the following post hoc comparisons.

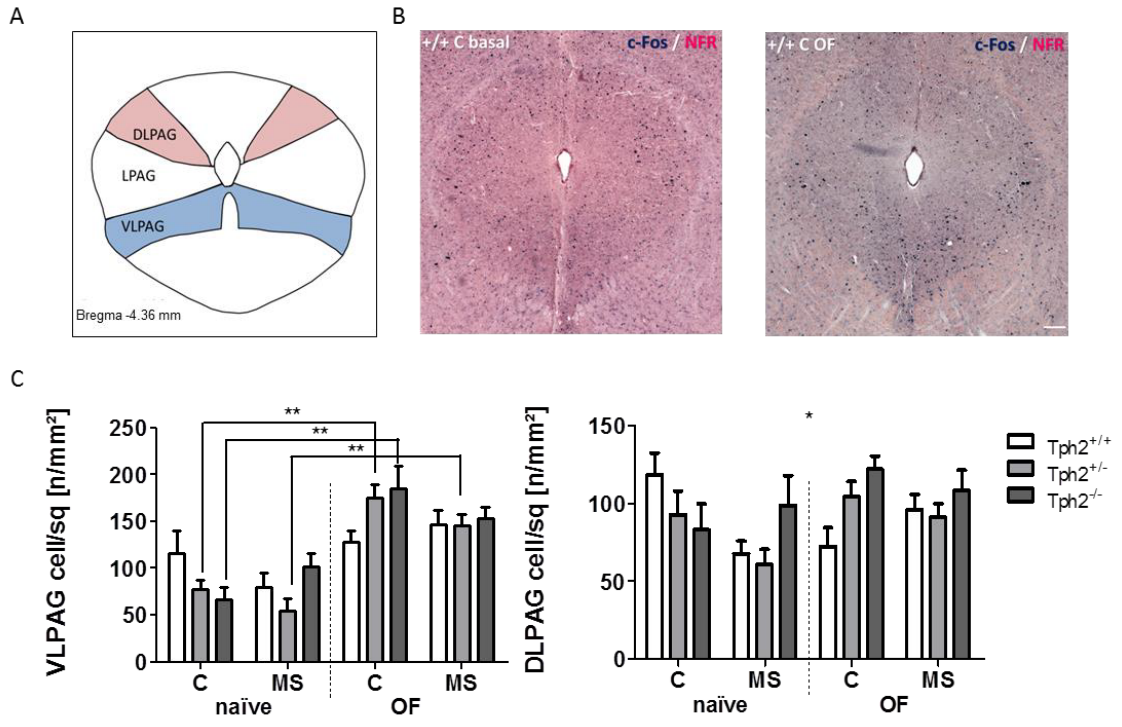

Figure 5 Neural activity in the ventrolateral and dorsolateral periaqueductal grey following open-field testing, as determined by c-Fos immunoreactivity. (A) Anatomical scheme depicting ventrolateral (VLPAG) and dorsolateral periaqueductal grey (DLPAG). (B) Representative histological section of the c-Fos immunostaining at the amygdala at Bregma level -4.4 mm (C) Behavioural testing in the open-field (OF) leads to a genotype-by-environment- by test-dependent activation in DLPAG and VLPAG ($p < 0.050$). Bars represent group means ± standard error (n = 4-9). *$p < 0.050$ and **$p < 0.010$ (Post hoc: t-test, Bonferroni).

Discussion

This study aimed at unravelling the implications of variations in 5-HT system function, in anxiety-related behaviours, in the context of adverse, early experiences, such as MS. As such, we observed pronounced differences between *Tph2* genotypes with regard to the effect of MS on behavioural performance in the OF and DLB test. Furthermore, several test-specific and *Tph2* genotype- and MS-dependent neural activation profiles were observed within various regions of the fear circuitries, as shown by c-Fos immunohistochemistry. Previous studies, investigating the effect of general constitutive brain 5-HT depletion on anxiety-related behaviours, were inconsistent (Mosienko et al. 2014 a), albeit a converging body of evidence suggested decreased innate anxiety and exaggerated fear displays (Mosienko *et al.*, 2012; Waider *et al.*, 2017). A more subtle 5-HT reduction, as for example in *Tph2*$^{+/-}$ animals was not linked to major changes in these behaviours (Mosienko et al. 2014 b). Most of the observed effects were found to be sex-dependent (Gutknecht *et al.*, 2015). For example, in contrast to *Tph2*$^{-/-}$ males, females did not show a hyperlocomotor phenotype. The results of the current study in female mice suggest effects of *Tph2* genotype and MS on behaviour and neural activation, dependent on the aversiveness of the context. On the one hand, *Tph2*$^{-/-}$ animals showed a lower exploratory activity in the light compartment of the DLB test, compared to *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ animals. This effect was only observed in offspring of the control group, while MS seemed to rescue the exploratory activity in *Tph2*$^{-/-}$ offspring. Moreover, as the exploratory activity in the dark compartment was unaffected by both *Tph2* genotype and MS, an effect on general activity can be excluded. On the other hand, in the aversive OF test lack of brain 5-HT did not affect behaviour in control offspring, but

was associated with panic-related escape behaviours in combination with MS. Following MS, *Tph2*$^{+/-}$ and *Tph2*$^{-/-}$ offspring started jumping against the walls during the second half of the test period. A similar effect was observed in the light compartment of the DLB, where, in particular, *Tph2*$^{-/-}$ offspring of MS dams jumped. Interestingly, an increased initial latency to locomotion was observed to be most pronounced in *Tph2*$^{-/-}$ MS offspring, as well. Thus, MS during early life seemed to shift behaviours towards a proactive more flight-related coping, dependent on the *Tph2* genotype, similar to observations in a recent study on male *Tph2*$^{-/-}$ mice, which showed higher levels of fear and flight behaviours in response to novelty exposure and aversive footshocks (Waider *et al.*, 2017). Diverse studies, investigating the effect of early adversity, found such unfavourable conditions to be related to increased susceptibility to panicogenic agents such as CO_2, in particular in female subjects (Genest *et al.*, 2004; Dumont, Biancardi and Kinkead, 2011). Furthermore, early adversity showed pronounced effects on the 5-HT system, leading to a decrease of *Tph2* mRNA in the VLPAG of female rats (Lukkes *et al.*, 2013). Moreover, MS-induced, elevated anxiety levels were concomitant with a change in *5-Htt* and *Tph2* mRNA, but not associated with an altered activation of DR 5-HT neurons (Gardner et al. 2005; Gardner et al. 2009 a; Gardner et al. 2009 b).

Indeed, Deakin and Graeff suggested distinct roles of 5-HT in defence mechanisms against aversive events (Deakin and Graeff, 1991). The pathways suggested to be involved in these defensive mechanisms can be traced back to distinct subnuclei of the raphe, the PVN of the hypothalamus and the PAG as well as diverse subnuclei of the amygdala, (Paul and Lowry, 2013; Paul *et al.*, 2014). In line with this, the panic-like and exaggerated fear responses that were observed in *Tph2*-deficient animals were associated with a dysfunctional amygdalo-dorsal raphe circuitry (Waider *et al.*, 2017). In the current study, we found that exposure to DLB, or OF, induced neural activation in all investigated subnuclei of the amygdala. The neural activation of the La was specific for the employed anxiety test, with a general activation following the DLB test, while the activation following OF testing was MS-specific. These observations suggest a MS-dependent, modulatory input, leading to an increased activation under acute, more aversive conditions and might be involved in the 5-HT-dependent mediation of fear/panic-related behaviours, while anxiety related-behaviours seemed independent from La neural activation. This notion is supported by the finding that neural activation in the La by DLB was correlated with both the rearing frequency in the dark and in the light compartment. In contrast, activation in Ce and BL were correlated only with the rearing frequency in the dark. In the BL, general neural activation was observed following the OF test, while neural activation following the DLB test was genotype-specific and only observable in *Tph2*$^{+/-}$ offspring, which could represent a compensatory effect of *Tph2*$^{+/-}$ offspring (Waider *et al.*, 2017). The BL was shown to be involved in the regulation of the anxiety-related manifestations (Hale, Hay-schmidt, Mikkelsen, Poulsen, Adriaan, *et al.*, 2008; Waider *et al.*, 2017). Following the activation of the general stress response, corticotrophin releasing hormone (CRH) was suggested to facilitate BL activation (Paul *et al.*, 2014), which could explain the proportionally higher activation of the BL upon exposure to the OF test in comparison to the more ambiguous DLB test. Like in the La, the biggest portion of BL neurons have been shown to be glutamatergic of nature, projecting towards other amygdalar nuclei, amongst others the Ce (Millhouse and DeOlmos, 1983).

In contrast to the effects observed in La and BL, we found comparable activation of the Ce following DLB and OF testing, independent of the *Tph2* genotype and MS. This is in contrast to the observed behavioural phenotype, but in line with a compensational mechanism, active in the La and BL of the amygdala. Because serotonergic projections, emerging from diverse raphe nuclei are known to affect a multitude of 5-HT receptors in the basolateral complex of the amygdala (Asan, Steinke and Lesch, 2013), MS and 5-HT genotype may have differential effects based on the serotonergic neuron subtype innervating the La or the BL. Indeed, exposure of male adult rats to OF increased the neuronal activation of a subregion of the dorsal raphe (DR) 5-HT neurons that project to the BL (Hale, Hay-schmidt, Mikkelsen, Poulsen, Adriaan, *et al.*, 2008). An activation of these neurons by anxiogenic substrates of diverse nature were positively correlated not only with the display of anxiety-related behaviours (Abrams *et al.*, 2005; Hale *et al.*, 2010), but also with the neuronal activity in a subset of GABAergic neurons within the BL (Hale *et al.*, 2010). Taken together, these data suggest that the altered La activity, related to OF testing in response to MS, might represent one component of the circuitry involved in regulating panic-related behaviours, dependent on early-life experiences, while amygdala signalling related to DLB testing seems to be dissociated from the anxiety-related effects observed in *Tph2*$^{-/-}$ control offspring. Moreover, recent findings on an alternative regulation of anxiety-related amygdala circuits, based on co-release of the neurotransmitter glutamate from the 5-HT terminals (Sengupta *et al.*, 2017) may explain this lack of differential activation in the amygdala by adverse, early conditions or the actual 5-HT deficiency.

Neuronal activation in the PVN appeared genotype-dependent for the DLB. Neither an effect of genotype, nor an MS effect was detected for OF. This might be an indicator for the more complex nature of the experienced stress in the DLB testing, integrating additional input from brain regions such as the Ce (Herman and Cullinan, 1997; Herman *et al.*, 2005) as well as brain stem structures such as the DR (Van de Kar, 1997). In line with this notion, serotonergic synapses have been identified on CRH cells in the PVN (Liposits, Phelix and Paull, 1987) and systemic (i.p.) administration of fluoxetine caused a significant increase in CRH concentrations in hypophysial portal plasma (Gibbs and Vale, 1983). The conditions of the OF might represent an unconditioned stressor, activating PVN secretion of CRH directly (Herman and Cullinan, 1997). This supports the idea that 5-HT is necessary for modulating CRH release of PVN neurons. Therefore, lifelong absence of 5-HT synthesis may be involved in the behavioural phenotype of *Tph2*$^{-/-}$ animals in the DLB compared to *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ counterparts through modulating PVN reactivity.

One brain region, particularly involved in the regulation of panic-related escape behaviour, is the VLPAG (Paul *et al.*, 2014). VLPAG serotonergic neurons were shown to project to the DLPAG, where they are suggested to inhibit panic-related escape responses. This suppression of panic-like behaviour is thought to be mediated via direct and indirect inhibitory actions of the 5-Ht1a and 2a receptors in the DLPAG, respectively (Pobbe and Zangrossi, 2005; Pobbe *et al.*, 2011). Indeed, the observation of panic-like escape behaviour of MS *Tph2*$^{-/-}$ and *Tph2*$^{+/-}$ offspring in the OF may be directly linked to an absence or reduction in 5-HT signalling from the VLPAG, within the DLPAG respectively. However, activation of the VLPAG in *Tph2*$^{-/-}$ and *Tph2*$^{+/-}$ mice was increased after OF, under control conditions, probably resembling a developmental effect of 5-HT deficiency on the involved cell subtypes in brain circuits, switching active and passive fear responses (Tovote 2016). This effect seemed to be absent

in *Tph2*$^{-/-}$ MS offspring, providing evidence for an interference of MS on coping mechanisms in the VLPAG in fully 5-HT-deficient offspring. Indeed, the VLPAG, including parts of the dorsolateral DR has been shown to receive input from CRH projections of the Ce (Paul *et al.*, 2014).

In conclusion, this study suggests, on the one hand, that panic-related escape behaviours in females are strongly associated with the synergistic interaction between brain 5-HT deficiency and MS. MS seems to take effect via altered La activity, thereby, potentially, altering the perceived aversiveness of the environment, while an interaction between MS and 5-HT deficiency in consequence was influencing VLPAG reactivity, which might represent the effector of panic-related behaviours. On the other hand, anxiety-related behaviours seemed to be strongly affected by 5-HT deficiency per se. This effect might be related to the observed decrease in PVN activity in *Tph2*$^{-/-}$ animals compared to *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ animals. Furthermore, we suggest alternative pathways, independent of 5-HT, compensating lifelong absence of 5-HT, or maternal separation, which may interact brain region-specifically, under acute challenge. However, to gain further insights into the molecular components of these regulations, further work towards identifying the exact nature of activated neurons is needed.

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Supplementary information

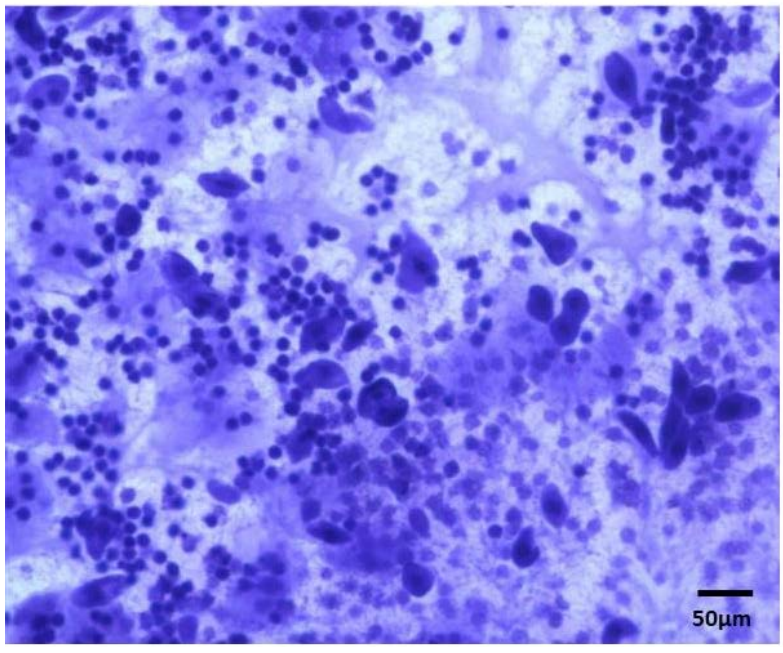
Breeding

All mating pairs were separated after 5 days and females that had shown a plug at least once were housed individually from then onwards. Females were weighed before mating and 4, 7 and 10 days after separation. Animals that did not show any weight gain over the first 10 days after separation were mated again, with different males. From 14 days after the separation onwards nests were checked for delivery.

Estrus-cycle determination

To allow comparability and avoid behavioural differences, caused by variations of estrogen levels, we determined the estrus-cycle around P41 and P55 and calculated the test day in such a way that animals would be in the post-ovulatory phase (met-estrus/di-estrus). To calculate the estrus cycle, a regular cycle was estimated and no difference between *Tph2*$^{-/-}$ and *Tph2*$^{+/+}$ females were assumed. To determine, the actual estrus during testing, the estrus was furthermore determined at the test day, prior to sacrifice. Indeed, more than 75% of the animals were in the post-ovulatory phase on the testing day.

For the vaginal lavage 100 µl of autoclaved ddH_2O was pipetted into the vaginal canal using autoclaved tips and a 100 µl pipette (Eppendorf, Hamburg Germany) without penetrating the tip into the vagina. Fluid was aspirated into the vaginal canal and withdrawn back into the tip. This was repeated about 20 times to obtain a sufficient cell number in the fluid. The vaginal lavage fluid was pipetted on a glass slide and dried at room temperature. For staining, the slides were placed into a jar containing 0,1% crystal violet stain (0,1 g crystal violet powder in 100 ml of ddH_2O) for 30 s and then carefully rinsed with ddH_2O to remove superfluous stain, put 1 min in 70% ethanol, followed by 96% ethanol, 100% ethanol and xylene. Then, slides were cover-slipped with Vitro Clud (NOVOGLAS, Langenbrinck, Germany; method in detail in (McLean *et al.*, 2012)). After staining, estrus cycle stage was determined using light microscopy (S 1).



S 1 Example for di-estrus, crystal violet staining

Dam and offspring physiological parameters

Dams were weighed at the day of their first mating and there was no difference between dams that eventually were subjected to the maternal separation (MS) paradigm and the control dams, for neither cohort. Furthermore, the number of pups did not differ significantly between groups, neither within nor between cohorts. In cohort 1 no difference between control and MS groups were observed neither in mothers' nor in pups' weight. In cohort 2 we found a decrease in dam weight in MS dams at P5 ($U = 5.7$, $p = 0.017$). The survival of pups was not influenced by the stress paradigm. From weaning onwards, a significant genotype effect on the weight of adult female offspring was observed independent of MS (for either cohort and at any time point (P25, P41 and P55), $p < 0.001$). Most prominently, post hoc analysis revealed that *Tph2*$^{-/-}$ female offspring of both cohorts had a lower body weight at all time-points (P25, P41 and P55 ($p < 0.025$; S2)) compared to *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ offspring. This effect was independent of MS. Furthermore, we observed an MS effect in cohort 2 *Tph2*$^{+/+}$ offspring at P41 and P55 ($p < 0.010$) and in cohort 1 *Tph2*$^{+/-}$ offspring at P55 ($U = 77.0$, $p = 0.020$), weighing more than non-stressed controls and in cohort 1 a difference between non-stressed control *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ offspring at P41 ($U = 65.0$, $p = 0.017$).

S 2 Table Group size and body-weight in offspring of control or maternal separation dams throughout life (postnatal day 25-55 (A-C)).

A)

condition	n	*Tph2*$^{+/+}$ P25±3			*Tph2*$^{+/-}$ P25±3			*Tph2*$^{-/-}$ P25±3		
cohort 1										
C	40	14.42	±	0.50	14.01	±	0.53	8.07	±	0.53
MS	49	14.84	±	0.31	14.40	±	0.35	9.20	±	0.41
cohort 2										
C	44	12.94	±	0.48	13.99	±	0.41	7.43	±	0.64
MS	42	13.35	±	0.51	12.85	±	0.50	6.97	±	0.52

B)

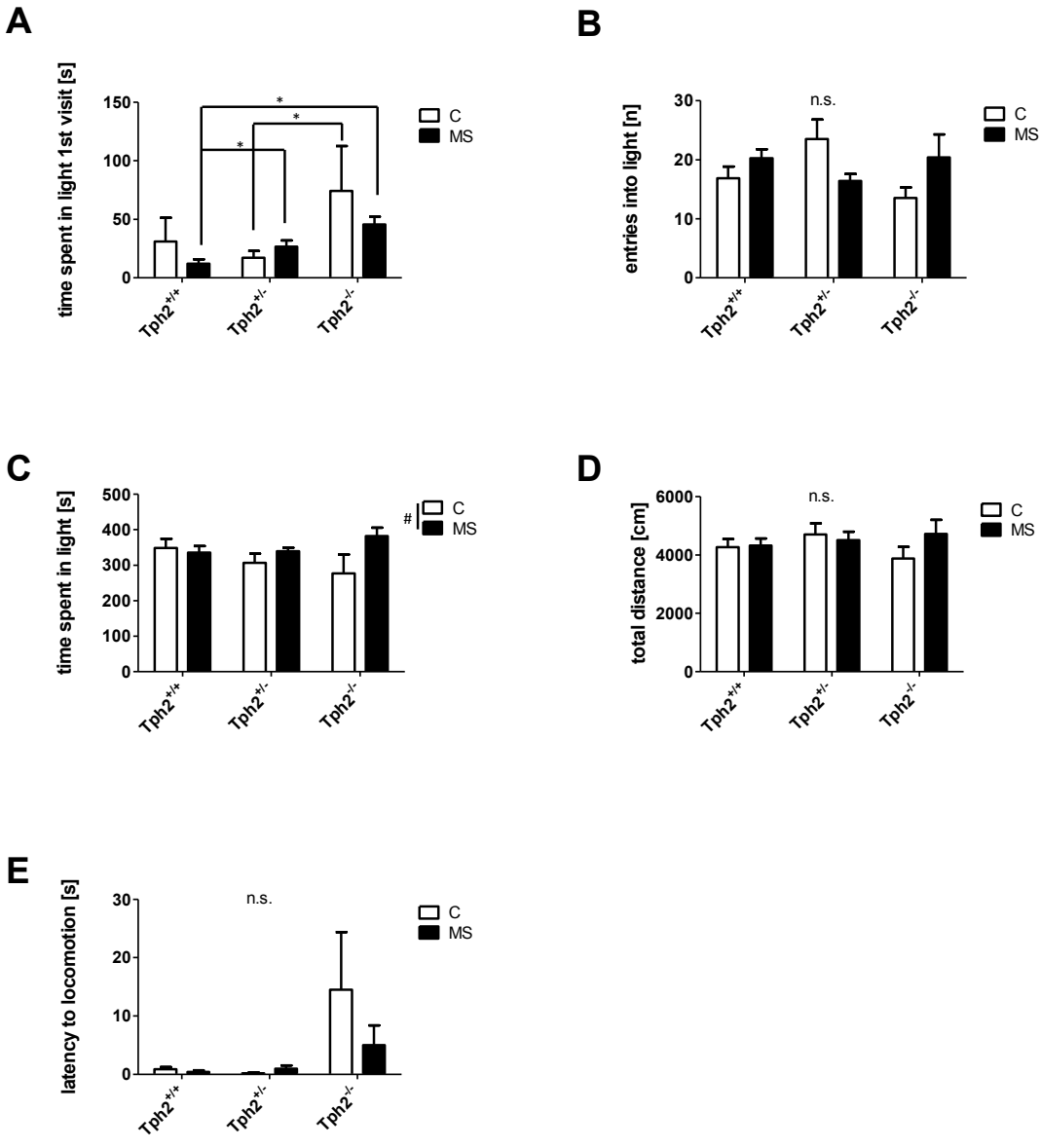
condition	n	*Tph2*$^{+/+}$ P41±7			*Tph2*$^{+/-}$ P41±7			*Tph2*$^{-/-}$ P41±7		
cohort 1										
C	40	19.26	±	0.28	18.66	±	0.47	15.90	±	0.44
MS	49	19.85	±	0.26	19.49	±	0.31	15.98	±	0.26
cohort 2										
C	44	16.24	±	0.44	18.33	±	0.57	12.26	±	1.01
MS	42	18.10	±	0.30	18.50	±	0.20	14.29	±	0.46

C)

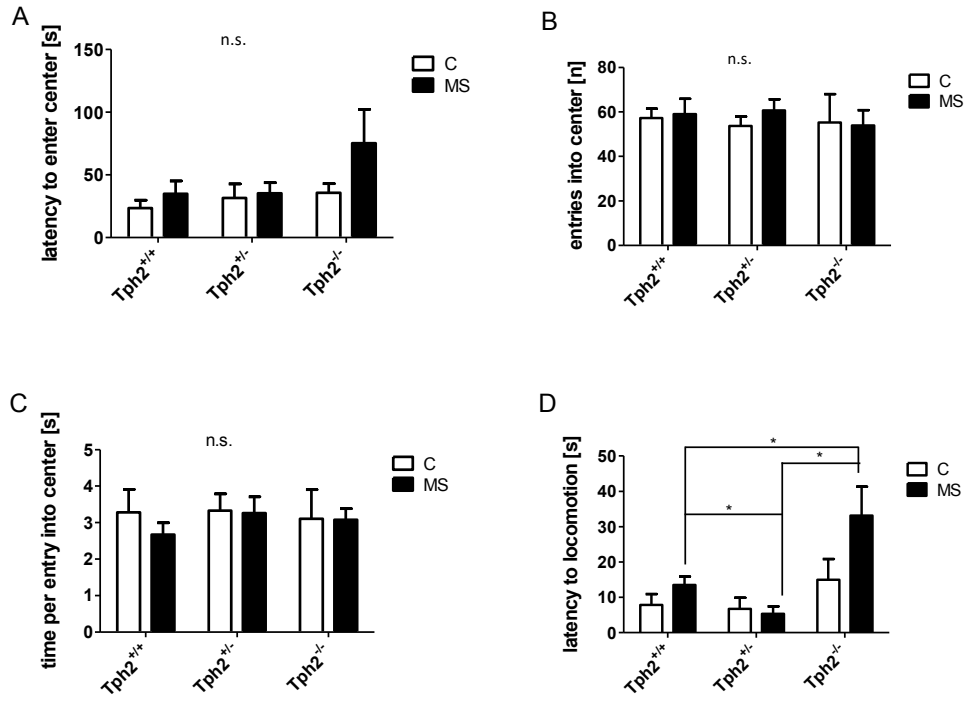
condition	n	*Tph2*$^{+/+}$ P55±7			*Tph2*$^{+/-}$ P55±7			*Tph2*$^{-/-}$ P55±7		
cohort 1										
C	40	20.78	±	0.40	19.78	±	0.43	18.47	±	0.39
MS	49	21.61	±	0.30	21.15	±	0.32	18.99	±	0.29
cohort 2										
C	44	20.27	±	0.31	21.31	±	0.39	18.56	±	0.57
MS	42	21.73	±	0.38	21.55	±	0.24	18.99	±	0.47

Full depletion of tryptophan hydroxylase 2 (*Tph2*) decreases body-weight when compared to *Tph2*$^{+/-}$ and *Tph2*$^{+/+}$ offspring independent of maternal separation (MS) ($p < 0.005$). In addition, MS increased the body-weight of offspring in cohort 2 at P41 and P55 and in cohort 1 *Tph2*$^{+/-}$ offspring at P55 ($p < 0.030$). Values represent group means ± standard errors, n = 9-17 per group. *$p < 0.050$ (Post hoc: Mann-Whitney U).

Offspring behaviour



S 3 Figure Distinct behavioural parameters measured during the dark-light box. (A) Time spent in light during first visit of the light compartment was affected by an interaction between tryptophan hydroxylase 2 (TPH2) deficiency and maternal separation (MS) ($p < 0.050$), with *Tph2*$^{+/+}$ that were exposed to MS spending less time during their first visit. (B) Number of entries into light compartment was not affected by TPH2 deficiency, MS or their interaction. (C) The effect of MS approached significance for the time spent light over the total testing period ($p < 0.100$) (D) The total distance animals moved during the testing period and (E) latency to locomotion did not differ significantly between groups. Values represent group means ± standard errors (n = 6-9). *$p < 0.050$, #$p < 0.100$ (Post hoc: Mann-Whitney U).



S 4 Figure Distinct anxiety-related behaviour parameters in the open-field. (A) Neither the latency to enter centre, nor (B) the number of entries into centre nor (C) the average time per entry spent in centre were affected by tryptophan hydroxylase 2 (TPH2) deficiency, maternal separation (MS) or their interaction. (D) *Tph2*$^{-/-}$ animals, subjected to MS, showed a longer latency to locomotion when compared to *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ animals of the same group ($p < 0.05$). Bars represent group means ± standard errors (n = 4-9). *$p < 0.050$ (Post hoc: Mann-Whitney U).

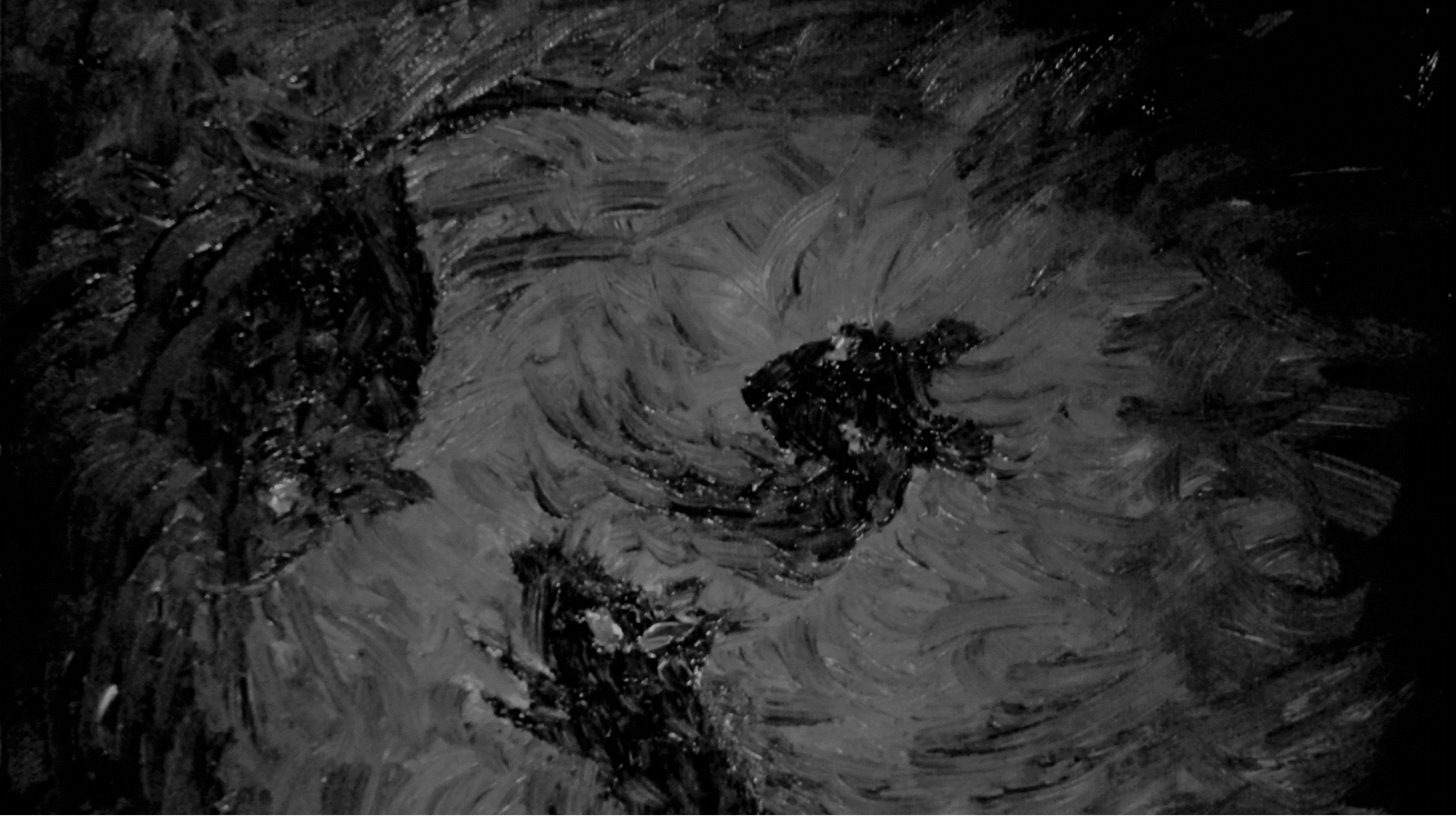
Brain volume measurements

Decrease in volume observed in several of the investigated regions (La and BL, $p < 0.050$) of *Tph2*$^{-/-}$ animals, with smaller La when compared to both *Tph2*$^{+/-}$ ($p = 0.003$) and *Tph2*$^{+/+}$ ($p = 0.007$) and a smaller BL when compared to *Tph2*$^{+/-}$ animals ($p = 0.018$) (S 5).

S 5 Table Total area measured for the basolateral, lateral and central amygdala, as well as the paraventricular nucleus of the hypothalamus.

	BL area [µm^2]		La area [µm^2]		Ce area [µm^2]		PVN area [µm^2]	
control *Tph2*$^{+/+}$	5975634±	183452	1700582±	63436	1234635±	50565	376992±	8212
MS *Tph2*$^{+/+}$	5764497±	196032	1981797±	106259	1357587±	67110	383501±	10721
control *Tph2*$^{+/-}$	5935762±	168944	1878449±	86102	1300084±	51494	377642±	11031
MS *Tph2*$^{+/-}$	6107297±	181416	1808567±	65555	1273798±	39792	373488±	6814
control *Tph2*$^{-/-}$	5625017±	185596	1485289±	52380	1192625±	28547	387087±	6794
MS *Tph2*$^{-/-}$	5463633±	144612	1642958±	85994	1221283±	46509	374516±	10286

Full tryptophan hydroxylase 2 (TPH2) deficiency reduced the observed volume in the lateral (La) and basolateral (BL) nuclei of the amygdala ($p < 0.020$). No effects were observed in the central (Ce) amygdala or the paraventricular nucleus (PVN). Values represent average area per brain region, per group ± standard error (n = 4-9).



Epigenetic priming of a SOX10 binding site associated with the myelin basic protein (*MBP*) promoter by prenatal stress in mouse and man

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Abstract

DNA methylation at the gene encoding myelin basic protein (*MBP*) has previously been associated with stress exposure *in utero* and its phenotypic consequences. The present study aimed to validate this involvement of MBP with early adversity on multiple levels, in humans and mice. In humans, *MBP* gene methylation was assessed in umbilical cord blood of infants, whose mothers were subject to comprehensive examination throughout pregnancy, using stress- and anxiety-related questionnaires and saliva cortisol measurements. For the study in mice, we employed a prenatal, maternal restraint stress paradigm (PS) in C57BL/6 dams that were impregnated by serotonin transporter (*5-Htt*)-deficient males. Subsequently, *Mbp* expression and methylation, in the hippocampus of adult, female offspring, as well as their association with behaviour, were investigated. In human infants, an increase of the maternal cortisol, indicated by the area under the curve, averaged over the whole pregnancy, was positively related to *MBP* gene methylation at one of two investigated CpG sites in female offspring. In mice, PS-induced, reduced weight gain during the stress period was associated with an increased methylation at a previously identified CpG site at the *Mbp* gene in *5-Htt*$^{+/+}$ but not in *5-Htt*$^{+/-}$ offspring. Thus, the predicted effect of the prenatal environment on *MBP* gene methylation was shown to be sensitive to developmental cues in both mouse and man and is most likely dependent on sex and variation in serotonin system functioning.

Introduction

Harmful events during development, such as stress exposure *in utero*, early neglect or childhood abuse have been identified as important contributors to an increased susceptibility to mental illness later in life (Widom, 1989; McCauley *et al.*, 1997; Carr *et al.*, 2013). Pioneering work in this field uncovered that experiences throughout development have the capacity to carry on into later-life to affect the stress response and through such emotion regulation (Thompson, 1957; Levine, 1967). The specific effects of adversity in later life depend on numerous factors, such as timing and duration of the previous stress exposure, genetic predisposition and the challenges met later in life (Lupien *et al.*, 2009). Several studies in humans and animals identified the reciprocal regulation of serotonin (5-hydroxytryptamine; 5-HT) system function and early-life stress exposure as a crucial mechanistic link in the aetiology of mental disorders. The associated reciprocal programming of the developing brain by early stress and 5-HT, was suggested to be mediated in particular via epigenetic modifications (Booij, Richard, *et al.*, 2015).

Over the last decade, a considerable number of studies have focussed on elucidating the molecular mechanisms that allow such dynamic regulations of the stable genome. Epigenetic modifications comprise a variety of modifications at the nucleosome-forming proteins called histones, as well as methylation and hydroxymethylation at the DNA strand itself. Utilizing DNA methylation measures from peripheral cells of blood or saliva, or samples of brain tissue, yielded a substantial body of evidence, linking methylation changes to the exposure to various forms of early-life adversity, and subsequent, pathological consequences (Klengel *et al.*, 2014). The extent to which the observed methylation patterns are reflective of the adverse circumstances, experienced early in life (verses or in addition to other experiences and life events), and whether they are causally related to physical and mental health and disease in later life, is unknown. Human studies on developmental stress are associated with unavoidable, confounding factors, which complicate the determination of specific events as causal factors for particular variations in brain functions and related behaviours. Animal models have thus become vital to study such relationships, allowing for a tighter control of variables, including the timing and duration of exposure to stress and other environmental variables, as well as the genetic background. Therefore, comparative approaches help to model neurobehavioural outcomes that translate across species and, might allow the identification of potential candidate mechanisms underlying these outcomes (Carlson, 2012). Regardless of the species, developmental stress typically modifies neural development, compromising behaviour in later life. However, some individuals display remarkable stress coping and unaffected adult behaviour. This dichotomy might be influenced by gene polymorphisms and epigenetic mechanisms affecting neuronal correlates. In a recent mouse study, we looked into the molecular mechanisms mediating long-term, 5-HT-dependent effects of gestational stress. Using a maternal, restraint stress paradigm (Behan *et al.*, 2011), applied in heterozygous serotonin transporter (*5-Htt*$^{+/-}$) -deficient mice (Bengel, Murphy and Andrews, 1998), we showed that the long-term behavioural effects of prenatal stress (PS) are partly dependent on the *5-Htt* genotype (van den Hove *et al.*, 2011). Molecular screening of the hippocampus of adult, female offspring revealed, furthermore, 5-HT- and stress-dependent effects on the DNA methylation profiles of numerous genes (Schraut *et al.*, 2014). One candidate of particular interest, i.e. the gene encoding myelin basic protein (*Mbp*), was found to be affected by the *5-Htt* genotype and its interaction with PS.

Next to the methylation profile, this interaction also affected the *Mbp* expression profile. Subsequent fine-mapping analysis identified 1 CpG site to be associated with anxiety-related behaviour (Schraut *et al.*, 2014). Numerous experiments in other groups were also able to relate 5-HT function and early stress to oligodendrocytes (OLs), and myelin (Ono *et al.*, 2008; Simpson *et al.*, 2011; Chetty *et al.*, 2014; Fan *et al.*, 2015). OLs are prominently involved in axonal sheath myelination (Simons and Nave, 2016) and known to be the main source of expression for the investigated myelin-associated candidate genes (Cahoy *et al.*, 2008). Following early weaning, a precocious myelination in the basolateral amygdala of male mice was observed (Ono *et al.*, 2008). The myelination was described using electron microscopy, revealing an increase in the number and a decrease in the diameter of myelinated axons in the anterior part of the basolateral amygdala in early-weaned male mice at 5 weeks of age. In contrast, in adult animals, stress as well as corticosterone exposure increased oligodendrogenesis and decreased neurogenesis in the dentate gyrus (DG) of the hippocampus (Chetty *et al.*, 2014). Treatment with selective 5-HT reuptake inhibitors (SSRIs), administered perinatally, evoked alterations of the myelination of callosal and somatosensory axons as well as interfered with OL soma morphology (Simpson *et al.*, 2011). In this study Simpson and colleagues, furthermore, showed a dose-dependent effect of 5-HT on OL survival. 5-HT treatment-induced death of OLs has been shown to be, at least in parts, mediated by the 5-HT2a receptor (Fan *et al.*, 2015).

In the present study, we aimed to validate the role of MBP as a modifiable factor in early-life stress. Next to further examining its role in animals, we aimed to translate these findings and validate its epigenetic regulation by early adversity in a cohort of human subjects, examined comprehensively, using questionnaires and saliva cortisol measurements throughout pregnancy.

Material and methods

Human cohort and procedures

The Prenatal Early Life Stress study (PELS) was approved by the ethical committee of the University Hospitals of Leuven, Belgium (n˚S51757). Consent of all participants was obtained in written from. In total, 80 pregnant women of the general population were recruited from the antenatal clinic of the University Hospital in Leuven, at around week 6–12 of gestation. Once per trimester, the women were asked to complete the following questionnaires: the pregnancy-related anxiety questionnaire (**PRAQ**) (Van Den Bergh *et al.*, 2005), the Edinburgh Depression Scale (**EDS**) (Cox, Holden and Sagovsky, 1987) and the Spielberger State Trait Anxiety Inventory (**STAI**) (Spielberger, Spielberger and D., 2010) and to collect saliva cortisol samples at two consecutive days: at awakening and 30 min, 4 h and 12 h later (Hompes *et al.*, 2013). In the current study, an average over the three sampling timepoints was calculated for all questionnaires, and, for the cortisol analysis, the average of the determined area under the curve (AUC) was calculated over all collected samples. Umbilical cord blood was taken at birth and, subsequently, DNA was extracted. Detailed information on sample collection, questionnaires and demographics have been previously published (Hompes *et al.*, 2012, 2013; Vangeel *et al.*, 2017) and all relevant information is also described in more detail in the supplementary information.

Animals and procedures in a rodent model of prenatal stress

The animal part of the present study was approved by the governmental department of Unterfranken, (Würzburg, Bavaria, Germany; Permit number: 55.2-2531.01-93/12) and all efforts were made to minimize animal numbers and suffering. *5-Htt*$^{+/-}$ mice (B6.129(Cg)-Slc6a4tm1Kpl/J (Bengel, Murphy and Andrews, 1998); ZEMM, Würzburg) and their wildtype (C57BL/6) siblings were exposed to PS or left undisturbed during gestation (embryonic days (E) 13 through 17) (Behan *et al.*, 2011). From 2 months of age onwards, behavioural screening of socio-emotional behaviours and stress reactivity was performed in female offspring. In brief, animals underwent behavioural screening starting with elevated plus maze (EPM) (Lister, 1987; Post *et al.*, 2011; Gutknecht *et al.*, 2015), followed by Porsolt swim test (PST) (Porsolt, Le Pichon and Jalfre, 1977; Behan *et al.*, 2011), and 3-chamber sociability test (3-CST) (Yang, Silverman and Crawley, 2011). Following behavioural testing the corticosterone response to restraint stress was investigated in plasma (Van den Hove, Steinbusch, *et al.*, 2006). Litters smaller than 5 pups were disregarded (Tanaka, 1998). Around P90 all animals intended for further molecular investigation, were sacrificed. Brains were harvested and stored at -80°C. Subsequently, the hippocampus was dissected, the tissue was powderised, blended and two equal portions were created from which RNA and DNA were extracted as described elsewhere (van den Hove *et al.*, 2011; Schraut *et al.*, 2014). All experimental procedures are described in more detail in the supplementary information.

Investigation of epigenetic regulation

DNA methylation was assessed by bisulfite conversion-based pyrosequencing. For the human samples 0.5 µg genomic DNA (gDNA), derived from umbilical cord blood were assessed for the DNA methylation profile near a transcription start site of the *MBP* gene. Bisulfite conversion of gDNA was performed using the EZ DNA methylation kit (Zymo Research, Irvine, CA, US) following the manufacturer's recommendations for Illumina. In the animal part of the study, the DNA methylation profile in the hippocampus, at a region analogue to the human site, was examined. This locus had been previously identified to be susceptible to environmental cues such as PS (Schraut *et al.*, 2014). Bisulfite conversion was performed using the EpiTect Fast Bisulfite Conversion kit (Qiagen, Hilden, Germany) and an input of 0.8 µg gDNA. For the pyro-sequencing analysis, amplicons of interest were amplified from bisulfite converted DNA (biDNA) using biotinylated primers, designed with the PyroMark Assay Design 2.0 software (Qiagen, see Supplementary Tables S 1 and S 3). Sequencing was performed using the PyroMark Q96 ID (Qiagen) as described elsewhere (Schraut *et al.*, 2014). Next to the DNA methylation profile, we also investigated the expression of two types of *Mbp* transcripts in the animals (van den Hove *et al.*, 2011; Schraut *et al.*, 2014). For a detailed description of the methods, used chemicals and primer sequences see supplementary information.

Statistical analysis

All data were collected in Microsoft Excel (Microsoft, Redmond, WA, US) and further exported and analysed in R (version 3.4.2; The R Foundation, Vienna, Austria) and RStudio (version 1.0.143; The Foundation for Open Access Statistics, Boston, MA, US). Extreme outliers were excluded from the analysis and log 10 transformation was performed to address skewed data. The effect of PS on relative dam weight gain, in mice, as well as the effect of gender on *MBP* gene methylation, in humans, was

analysed, using independent t-test. Significant statistical level was set at $p < 0.050$, unless otherwise specified, when multiple testing correction was required.

In humans, a multiple-linear regression model was used to examine the impact of maternal PRAQ, STAI and EDS scores as well as saliva cortisol AUC on variations in methylation levels at the *MBP* gene in the offspring. The models were adjusted for a set of covariates, including gestational age at birth, infant *5-HTT* gene-linked polymorphic region (5-HTTLPR) genotype (s/s and combined s/l and l/l), smoking and alcohol use during pregnancy. Based on the number of tested predictors the level for significance was set to $\alpha < 0.0125$.

To analyse the effect of PS on the methylation levels of 5 CpGs, associated with the *Mbp* gene promoter, as well as gene expression of 2 isoforms in mice, a multiple-linear regression model was used. PS, dam weight increase [(E17-E13)/E17], offspring *5-Htt* genotype as well as the interaction of PS with offspring genotype and dam weight increase with offspring genotype were used as predictors of *Mbp* gene methylation and expression. A hierarchical linear model was applied when the increase in dam weight or the interaction of dam weight increase was used as predictor. The hierarchical linear regression model allowed us to add the litter size as cluster and to control for the effect of litter size on the weight changes in dams. For this regression analysis the level of significance was set below 0.010. Furthermore, the predictive value of DNA methylation and gene expression regarding several, relevant behavioural and physiological measures in adult offspring was investigated, dependent on the *5-Htt* genotype and condition. As dependent variables we investigated 8 parameters (EPM: distance, time and visits in open arms, 3-CST: time in chamber with target animal, PST: distance covered and corticosterone response: baseline, stress and differential corticosterone). The level of significance was set as $\alpha < 0.007$ based on the number of CpGs and *Mbp* isoforms investigated (5 CpGs and 2 isoforms).

Based on the study design (van den Hove *et al.*, 2011) and on independent reports on sex-specific methylation in humans (El-Maarri *et al.*, 2007; Zhang *et al.*, 2011), the human sample was analysed separately for male and female offspring.

Results

Maternal stress during pregnancy and umbilical cord blood methylation in human infants

The previously reported sex-specific methylation was only moderate at the two CpGs investigated in this human sample, with a slightly higher methylation at CpG1 in males than in females ($p = 0.078$). Multiple regression analysis stratified per gender revealed a positive association of maternal cortisol AUC during pregnancy with methylation at CpG1 in female infants (Est = 0.009, $t = 2.66$, $p = 0.012$), but not in male infants (Est = 0.002, $t = 0.81$, $p = 0.424$; Figure 1). None of the other investigated measures of maternal distress in pregnancy were found to be associated with DNA methylation in either male or female offspring.

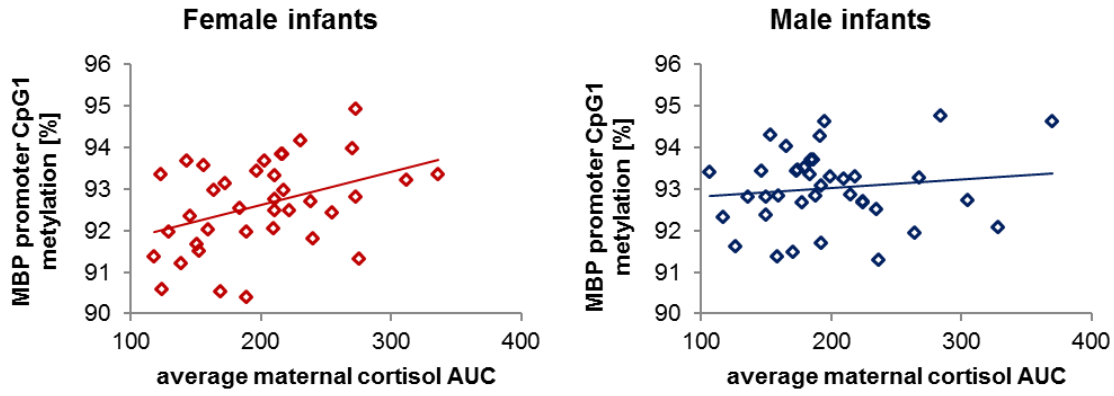

Figure 1 Association between average cortisol, indicated by the area under the curve (AUC), in mothers during pregnancy and infant myelin basic protein (*MBP*) gene methylation at CpG1. Multiple regression analysis revealed a positive association between the maternal cortisol AUC, averaged over 3 time-points during pregnancy, and the DNA methylation at the *MBP* CpG1, which is located close to a regulatory binding site near the transcription start site (p = 0.012). Female and male offspring were investigated separately, because of the experimental design of the animal studies (n = 37 females, n = 38 males).

Prenatal maternal restraint stress and Mbp gene methylation levels in mouse offspring hippocampus

In stressed dams, relative body-weight gain was lower in comparison to unstressed dams (p < 0.001). Multiple-linear regression analysis in the offspring revealed no association of the maternal condition, relative dam weight gain or offspring *5-Htt* genotype with either *Mbp* expression or *Mbp* gene methylation. However, an interaction model of relative dam weight gain with the offspring *5-Htt* genotype revealed a significant association of maternal weight gain with DNA methylation at *Mbp* CpG12, dependent on the *5-Htt* genotype (Est = 0.903, t = 2.82, p = 0.009; Figure 2). *Mbp* gene methylation and expression of either isoform were not associated.

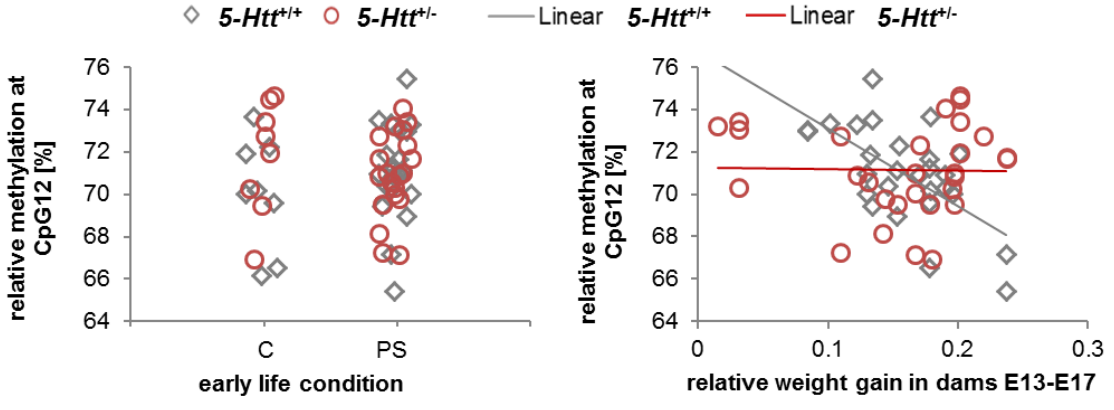

Figure 2 Association between prenatal stress (PS) and relative weight gain in dams between the day E13 (before) and E17 (after) exposure to a stress paradigm and the myelin basic protein (*Mbp*) CpG12 methylation in female offspring. There was no association of PS and *Mbp* gene methylation at CpG12 (control (C): n = 16, PS: n = 41). Relative weight gain of dams during the stress period was associated with *Mbp* gene methylation in the hippocampus of adult, female offspring, dependent on their serotonin transporter (*5-Htt*) gene variant (p < 0.010).

The effects of *Mbp* gene methylation and expression on the observed behaviours are depicted in supplementary Table S 4. None of the investigated parameters reached the level of statistical significance.

Discussion

This study aimed to validate a recently uncovered candidate gene, *Mbp*, which was shown to be differentially regulated by DNA methylation, dependent on the interaction of *5-Htt* genotype and the prenatal environment in mouse hippocampus. Overall, the predicted effect of the prenatal environment on *MBP* gene methylation was shown to be sensitive to developmental cues, both in the brain of adult mice, previously exposed to PS, and the blood of human infants, whose mothers were subject to comprehensive examination throughout pregnancy.

In both human and mouse offspring, the affected CpGs were associated with a predicted binding site of OL transcription factor, i.e. sex determining region Y-box 10 (Sox10). SOX10 is known to be one of the essential myelin transcription factors in the central nervous system, where it induces terminal OL differentiation. It is expressed already in OL progenitors, and directly induces other transcription factors necessary for OL differentiation (Hornig *et al.*, 2013). SOX10 was shown to enhance *Mbp* gene promoter activity in a tissue-dependent manner (Wei, Miskimins and Miskimins, 2004). The interactions of Sox10 and other transcription factors such as specificity protein 1 (SP1), OL lineage transcription factors (OLIGs) 1 and 2 or hairy/enhancer of split 5 (HES5) might determine the expression of *Mbp* in a highly specific manner (Weider, Reiprich and Wegner, 2013). Thus, methylation at the SOX10 binding-site might represent a switch for facilitating or inhibiting further activation of the *Mbp* gene promoter. In line with this notion, SOX10 was found to interact with epigenetic regulators and influence phenotypic outcome, dependent on this interaction (Weider, Reiprich and Wegner, 2013). However, it has to be mentioned that the investigated SOX10 binding site has not been validated *in vivo*.

The *Mbp* gene comprises a complex locus at chromosome 18 in the mouse and human genome with three identified transcription start sites, giving rise to numerous isoforms (Harauz and Boggs, 2013). Next to the so called ‘classic’ isoforms that arise from the transcription start site 3, the *Mbp* gene locus harbours several additional exons, encoding golli-specific isoforms (Campagnoni *et al.*, 1993). These golli-specific isoforms are not only expressed in OLs but also in neuronal cells (Landry *et al.*, 1996). *Mbp* isoforms, arising from the classic locus are exclusively found in OLs in the central nervous system and their functions range from forming compact myelin to modulating voltage-operated calcium channels (Harauz and Boggs, 2013). Mouse lines, deficient for MBP (shiverer, shi), show severe hypomyelination, concomitant with seizures and a decreased lifespan (for review see (Jacobs, 2005)). Milder forms of MBP deficiency (shi^{mld}) were associated with poorly compacted and sparse myelin. In addition, altered myelination and imbalanced expression of myelin-related genes were associated with a wide range of behavioural deficits. For example, PS increased anxiety in the open-field test concomitant with a decrease in MBP in the brain (Bennett *et al.*, 2015) and it induced depressive-like behaviour and decreased the number of glial cells in the hippocampus of female mice (Behan *et al.*, 2011). Precocious myelination of the basolateral amygdala, induced by early weaning, was associated with increased anxiety (Ono *et al.*, 2008). Such behavioural effects of dysregulated myelin are suggested to be related to altered neurotransmitter signalling in the hippocampus (Schraut *et al.*, 2014). As the hippocampus is a brain region relevant for emotion regulation, amongst others through GABAergic signalling, disturbances in myelin-related signalling may cause alterations in anxiety-related and other affective behaviours (Trent and Menard, 2010; McEown and Treit, 2013; Zhang *et al.*, 2014). In human major depression subjects, hippocampal volume, which might be indicative of an

altered myelination (Cobb *et al.*, 2013), had been found to be related to early adversity, dependent on the 5-HTTPLR (Frodl *et al.*, 2010). Furthermore, hippocampal volume was strongly associated with the DNA methylation at a regulatory-relevant locus of the *5-Htt* gene, which had previously been associated with *in vivo* measures of brain 5-HT synthesis in the blood, suggesting a potential route of effect of early adversity in the context of altered 5-HT system functioning (Booij, Szyf, *et al.*, 2015).

In the current study, maternal cortisol, averaged over the whole pregnancy, was associated with DNA methylation at one of the two investigated *MBP* CpG sites, i.e. CpG1, in human infants. Higher *MBP* CpG1 methylation was found in infants of mothers with higher cortisol levels throughout pregnancy. This might represent a direct effect of maternal cortisol on the epigenome. Indeed, glucocorticoids during sensitive periods have been associated with epigenetic programming. Both hypo- and hyper-methylation were observed in a targeted manner (Thomassin *et al.*, 2001; Niwa *et al.*, 2013). In neural stem cells, artificial glucocorticoid induced genome-wide hypo-methylation, associated with an altered expression of genes involved in epigenetic functioning, such as DNA methyltransferase 3a and Tet methylcytosine dioxygenase 3 (Bose *et al.*, 2015). Therefore, maternal cortisol seems to be a key factor in modulating the epigenetic landscape during development. Furthermore, as in the human part of the current study, the investigated umbilical cord blood was taken at birth, the observed effects on *MBP* gene methylation in the human subjects are suggested to be exclusively related to the prenatal environment.

Interestingly, the effect of maternal cortisol seemed to be sex-specific and was only apparent in female infants. Sex-specific consequences of adverse events on mental health have been observed in numerous studies (Buss *et al.*, 2012; Glasheen *et al.*, 2013). During foetal development, stress-induced programming might interfere with the predetermined, neurodevelopmental pathways (Bale and Epperson, 2015). Therefore, dependent on the period and nature of stress exposure, male and/or female individuals might show an increased risk for the onset of a wide range of mental disorders. One underlying factor in this sex-specific programming might be a distinct epigenetic regulation. Generally, DNA methylation in human blood has been found to be globally increased in males, with the exception of imprinted regions (El-Maarri *et al.*, 2007; Zhang *et al.*, 2011). During human foetal brain development, broad changes in DNA methylation can be observed (Spiers *et al.*, 2015). These changes have been found to be subject to partially sex-specific developmental trajectories. Amongst regions with sex-specific profiles were also autosomal regions, which remained sex-specific into adulthood. In another study, reporting previous findings concerning the same human cohort, as investigated in the current study, a PRAQ questionnaire subscale was found to be associated with DNA methylation at the GABA-B receptor subunit 1 gene (Vangeel *et al.*, 2017). This effect was observed in male infants exclusively, supporting the sex-specific mediation of disturbances of distinct nature by altering the epigenetic landscape *in utero*.

Similarly, in animal studies, sex-specific consequences of adverse events have been observed, with regard to affective as well as socio-emotional behaviours (Franklin *et al.*, 2011; Hiroi *et al.*, 2016), the HPA axis (McCormick *et al.*, 1995; García-Cáceres *et al.*, 2010), and the 5-HT system (Hiroi *et al.*, 2016). In accordance with these studies, we observed, in a previous animal study, effects of PS, predominantly, in female offspring (van den Hove *et al.*, 2011). Therefore, we had investigated only

female offspring in the animal part of the current study. Interestingly, we observed no association of the stress paradigm with *Mbp* gene methylation levels at a set of previously determined CpGs (Schraut *et al.*, 2014) in the hippocampus of adult female offspring. However, this might not be unexpected, as in comparison to the human study, where an actual biomarker and effector of maternal stress was found to alter *MBP* gene methylation, mice were exposed to a stress paradigm, allowing a distinction only between exposed and non-exposed individuals, neglecting inter-individual variability in stress responsiveness. Inter-individual variability between experimental animals was only recently discussed as a vital factor that has been neglected throughout research (de Boer, Buwalda and Koolhaas, 2016). Work in our own group revealed differential susceptibility of female offspring to PS, rendering a portion of stressed animals resilient in adulthood, while others showed increased depressive-like behaviour (Jakob *et al.*, 2014). The molecular mechanisms, underlying the observed resistance to PS, seemed to be dependent on offspring *5-Htt* genotype, suggesting distinct compensational processes. Next to the offspring genotype, another potential mediator of offspring phenotype is maternal stress susceptibility (Meaney, 2001). Offspring of non-responsive (resilient) stressed mothers might be exposed to different programming effects *in utero*, when compared to offspring of susceptible mothers, strongly affected by the stressor.

In an attempt to correct for maternal susceptibility, we investigated the association between weight gain during the period of stress exposure, a measure, which has been repeatedly reported as indicative of the experienced stress (Rybkin *et al.*, 1997; Harris *et al.*, 2002, 2006; van den Hove *et al.*, 2011; Jeong, Lee and Kang, 2013), and *Mbp* gene methylation. Using the relative weight gain, we identified DNA methylation changes to be associated with the weight gain of dams between E13 and E17, i.e. during PS exposure, dependent on the offspring *5-Htt* genotype. Lower weight gain in dams was associated with higher methylation at the *Mbp* CpG12, which indicates a positive association between severity of the experienced stress and DNA methylation and was similar to the observations in human, female infants. This association was significantly stronger in *5-Htt*$^{+/+}$ offspring, which is in accordance with multiple lines of evidence, converging to support the idea of 5-HT-dependent, epigenetic modulation (Homberg and van den Hove, 2012). On the one hand, various studies in humans, non-human primates and rodents, found effects of variations in 5-HT system function on adult emotion regulation, in particular, in interaction with aversive experiences in early-life (e.g.(Bennett *et al.*, 2002; Caspi *et al.*, 2002, 2003; Champoux *et al.*, 2002; van den Hove *et al.*, 2011; Sachs *et al.*, 2015; Wong *et al.*, 2015)). On the other hand, functioning of the 5-HT system has been shown to be involved in epigenetic modifications by e.g. facilitating the recruitment of chromatin remodelling factors and transcription factors such as cAMP-response- element-binding-protein-binding protein (CBP) and early growth response protein 1 (Egr1) (Hellstrom *et al.*, 2012).

As mentioned in the introduction, we discovered in a previous study that *Mbp* gene methylation was altered in a gene-by-environment interaction-specific manner (Schraut *et al.*, 2014). Subsequent fine-mapping identified in particular CpG12, to be affected, dependent on the *5-Htt* genotype and PS. However, while in the previous study CpG12 methylation was decreased in the group exposed to PS, in the current study the effect of lower weight gain, indicative of stress, was associated with an increase in DNA methylation. In both studies the effects were only observed in *5-Htt*$^{+/+}$ offspring. In light of the previously discussed potential influence of maternal stress susceptibility, these results are not

unexpected, as, in the previous study, dams were carriers of the heterozygous ablation of the *5-Htt* gene, while in the current study mothers were wildtype C57BL/6 mice. The *5-Htt* genotype has generally been associated with differential stress reactivity and HPA axis function (reviewed in (Murphy and Lesch, 2008)). Altered stress reactivity might be indicative of a differential susceptibility towards exposure to restraint stress during pregnancy. Thus, maternal susceptibility might be regulated by life experiences as well as by genetic predisposition. In turn and in interaction, both maternal and offspring *5-Htt* genotype might represent a basis for the effects of developmental programming (Jones *et al.*, 2010; Schraut *et al.*, 2014). Furthermore, in the previous study, *Mbp* CpG12 methylation was associated with *Mbp* expression and anxiety-related behaviours (Schraut *et al.*, 2014), while in the current study *Mbp* gene methylation was not statistically significantly correlated with *Mbp* expression or behaviour. This discrepancy of programming and expression as well as behavioural outcome, dependent on the maternal *5-Htt* genotype, is supporting the modulatory role that 5-HT might play in shaping the epigenetic landscape and, through such, indirectly gene expression, further. In addition to effects on the maternal susceptibility during pregnancy, the maternal genotype has also been shown to affect postnatal, maternal behaviour, and, consequently, might induce further regulation in the offspring (Francis *et al.*, 1999; de Souza *et al.*, 2013; Angoa-Pérez and Kane, 2014). Thus, a complex interaction of early life stress, functioning of the maternal and offspring 5-HT systems, and emerging long-term consequences might exert an intricate effect on DNA methylation, gene expression and behaviour.

Taken together, results of the current study suggest that in humans, *MBP* gene methylation in female infants is susceptible to the exposure to maternal cortisol in a targeted fashion. In mice a similar relation was observed with dam weight gain and *Mbp* gene methylation in *5-Htt*$^{+/+}$ female offspring. Further investigation of the association of methylation with gene expression and behaviour in offspring of stressed wildtype dams, in the light of previous findings (Schraut *et al.*, 2014), suggest that the observed changes in *Mbp* gene methylation represent most likely an indirect programming, acting e.g. permissive or inhibitory to, or in combination with further regulation. This suggestion is further confirmed by the observation that, altered *MBP* gene methylation was observed in blood of human infants in a similar fashion, as observed in the rodent hippocampus. In conclusion, the current study confirmed *MBP* gene methylation and, consequently, myelination as potential targets of environmental programming during the prenatal period, in particular in female *foeti*, and, furthermore, underlined the essential role of 5-HT system functioning therein. Our findings furthermore stress the importance of inter-individual variability in animal studies and suggest for the future a more thorough investigation of maternal phenotypes, to account for potential effects of maternal stress susceptibility.

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Supplementary information

Human cohort and procedures

The Prenatal Early Life Stress (PELS) study was approved by the ethical committee of the University Hospitals of Leuven Belgium (n˚S51757). All participants consented in written from. In total, 80 pregnant women of the general population were recruited from the antenatal clinic of the University Hospital in Leuven, Belgium, at around week 6-12 of gestation. Detailed information on sample collection questionnaires and demographics have been published previously (Hompes *et al.*, 2012, 2013; Vangeel *et al.*, 2017).

HPA axis activity assessment

Maternal cortisol was determined once per trimester at two consecutive days: at awakening, and 30 min, and 4 h and 12 h later. The cortisol was measured using the High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (ELISA kit, Salimetrics, Europe). Subsequently, the area under the curve (AUC) was calculated per day. In the current study, an average of the AUC, over all time-points during pregnancy, was investigated (for detailed description see (Hompes *et al.*, 2013)).

Questionnaires and behaviour

Psychological well-being in pregnancy was assessed by means of a number of self-report-based postal questionnaires that were gathered each trimester. More specifically, the pregnancy-related anxiety questionnaire **(PRAQ)** as described in (Van Den Bergh *et al.*, 2005), was used to measure specific fears and worries related to the participant's pregnancy. In a previous publication, concerning the cohort investigated in this study, several related questionnaires were performed and confirmed the PRAQ as a good representation of maternal state during pregnancy, displaying a good internal reliability (Cronbach's $\alpha \geq 0.95$) (Hompes *et al.*, 2013; Vangeel *et al.*, 2017). Next to the PRAQ, two other self-report questionnaires on psychological well-being were surveyed during each trimester of the pregnancy. A prenatal depression score was measured using the Edinburgh Depression Scale (EDS), which comprises 10 items with scores between 0 and 3 (Cox, Holden and Sagovsky, 1987). Furthermore, state-anxiety was determined using the Spielberger State Trait Anxiety Inventory (STAI) (Spielberger, Spielberger and D., 2010). In EDS as well as in PRAQ and STAI, higher scores indicate higher maternal depression or anxiety, respectively. None of the included mothers showed pathological levels in any of the questionnaires.

DNA methylation analysis

Before bisulfite-conversion, genomic DNA (gDNA), derived from cord blood, was quantified, using the Nanodrop (Thermo Fisher Scientific, Waltham, MA, US). Subsequently, 500 ng gDNA in 45 µl 1x TE was subjected to bisulfite treatment using the EZ DNA methylation kit (Zymo Research, Irvine, CA, US) following the manufacturer's recommendations for Illumina [(95°C for 30 s and 50°C for 60 min) x 16 cycles and then held at 4°C]. Subsequently, samples were loaded on binding plates and washed several times followed by a desulphonation step for 20 min at room temperature (RT). Following further washing steps, samples were eluted with 47 µl elution buffer, aliquoted and stored at -20°C.

For the pyrosequencing analysis, amplicons of interest were amplified from bisulfite converted DNA (biDNA) using biotinylated primers designed with the PyroMark Assay Design 2.0 software (Qiagen, Hilden, Germany) (Primers: **Table S 1**).

S 1 Table Primer pairs used for the bisulfite-conversion-based pyrosequencing analysis of myelin basic protein (*MBP*) in humans.

Name	modifications	Sequence	Product size [bp]
Pre-PCR primer			
hu_Mbp_F		TTTATAGGGGAAGAGTTGTTTTAGTATTAT	138
hu_Mbp_R	Biotin	AACTCTAAACACCATTCCAAT	
Sequencing primer			
hu_Mbp_Seq		CTAAACACCATTCCAATC	

The Primer set, used in the current study is covering a region analogue to a region that was observed to be differentially methylated dependent on an interaction of 5-HTT and PS, in previous work in mice (Schraut *et al.*, 2014). As the investigated region is not homologous between man and mouse, we identified a region in a similar position, as when compared to the mouse genome, that comprises, just like the CpG site identified as most significantly regulated in mice, a Sox10 binding site:

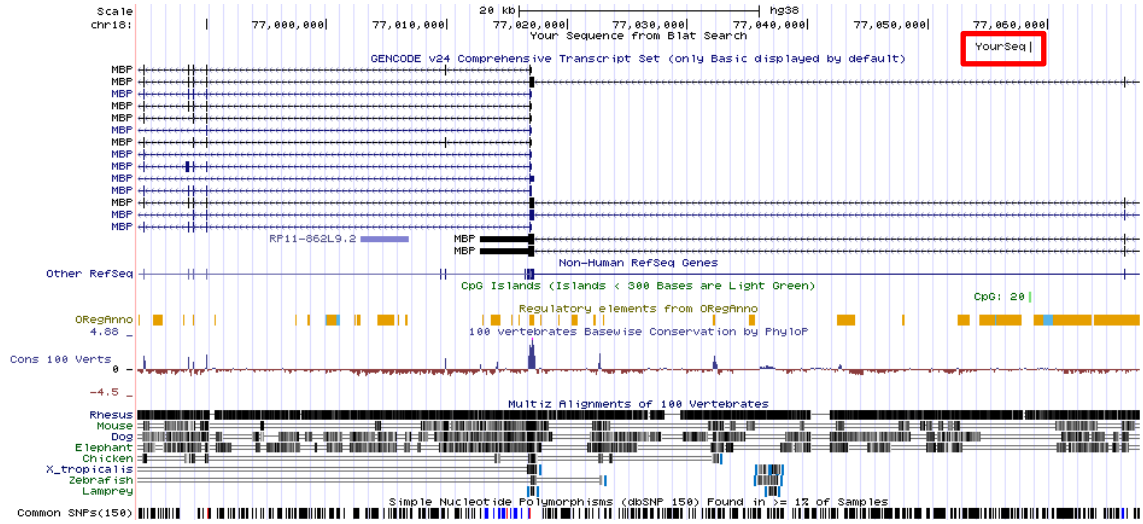
1 putative sites were predicted with these settings (80%) in sequence named **gi|224589809 : c74770620 - 74770323 (GRCh37/hg37)**

Model ID	Model name	Score	Relative score	Start	End	Strand	Predicted sequence
MA0442.1	SOX10	5.281	0.838899639980467	34	39	1	CCGTGT

Comment: This type of analysis has a high sensitivity but abysmal selectivity. In other words: while true functional will be detected in most cases, most predictions will correspond to sites bound in vitro but with no function in vivo. A number of additional constraints of the analysis can improve the prediction; phylogenetic footprinting is the most common. We recommend using the ConSite service, which uses the JASPAR datasets.

The review Nat Rev Genet. 2004 Apr;5(4):276-87 gives a comprehensive overview of transcription binding site prediction.

UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly



Your Seq:
chr18:77,058,522-77,058,660 (GRCh38; Chr 18: 74,770,479-74,770,616 GRCh37)
aaatggctctgggcaccattccagtc>>>actgc$_1$ggcc$_2$gtgttccaagtgcgtccacgatttcgtttcttaaagtgctgtttctgttgctcaggat
tctgcacgcgggggcgggggtggtgctggaacagctcgtcccctgtgggcgtcgcatcagccggtgggggctggggtcgtgcccgggcctg
gggtcgtgcctggggctggggtctgccggaggccgctccctcccgtggctggcgagctgtgcggggcgttggctgggtccggccgggctgtc
agcctgaactggtgccctgggt

- SOX10-binding sites
- CG: analysed CpGsites 1-2
- **PrePCR primer**
- **Seq primer>>>**

PCR amplifications for the pre-PCR were performed using the PyroMark PCR kit (Qiagen) and 2 µl biDNA template/reaction in a standard PCR-cycler (an initial enzyme activation for 15 min at 94°C, followed by 30 s at 95°C denaturation, 30 s at the primers' annealing temperature, and 30 s at 72°C for elongation, repeated for 45 times and a final 4 min at 72°C). Of the PCR product, 10 µl were used as input for the actual pyrosequencing reaction. To perform pyrosequencing the PCR product first had to undergo a cleaning process, during which one of the strands was washed off, while the strand with the biotinylated primer was further processed (in case of the hu_Mbp primers the reverse primer, see Table S 1). For this purpose, the biotinylated primer was bound to streptavidin sepharose High Performance beads (34 µm, GE Healthcare, Freiburg, Germany). The clean-up process was performed using the PyroMark Q96 Vacuum Workstation (Qiagen), according to the manufacturer's instructions. Following clean-up, the, now, single-stranded PCR templates were sequenced on the PyroMark Q96 ID (Qiagen) using the PyroMark Gold Q96 CDT Reagents kit (Qiagen) and sequencing primers (PyroMark Assay Design 2.0 software; Qiagen, S 1). Pyrosequencing results were analysed using the PyroMark CpG software (Qiagen).

For quality control purposes, we included unmethylated (0%), 50% methylated and fully (100%) methylated samples and no-template controls in the bisulfite-conversion, as well as in the sequencing runs. If the methylation levels of the controls were not identified correctly, this could indicate a bias in PCR amplification of biDNA, contamination, or incomplete bisulfite-conversion. All samples were run in 4 replicates, for which a deviation of 5% was accepted. The mean value of the 4 replicates was used for analysis. Samples that did not pass quality control were repeated or excluded.

Animals and procedures in a rodent model of prenatal stress

The animal part of the present study was approved by the governmental department of Unterfranken, (Würzburg, Bavaria, Germany; Permit number: 55.2-2531.01-93/12) and all efforts were made to minimize animal numbers and suffering. For breeding of the experimental animals, male *5-Htt*$^{+/-}$ mice B6.129(Cg)-Slc6a4tm1Kpl/J of the ZEMM, breeding facility, Würzburg were put together with two WT female mice (Charles River, Sulzfeld, Germany) each. Males were individually housed under 14 h/10 h light-dark cycle with lights on at 7AM and lights off at 9PM, at 21±1°C, with a maximum humidity of 55% prior to mating. Standard rodent chow and water were available *ad libitum*. Females were allowed to adapt to the housing conditions for at least 2 weeks upon arrival. From the moment the breeding

pairs were put together, females were tested for vaginal plugs daily. Following a positive plug control, referred to as embryonic day 0 (E0), females were housed individually. Body-weight of pregnant females was determined at E0, E13 and E17.

Prenatal stress

From E13 to E17 a subset of the pregnant females was subjected to restraint stress for 45 min, three times per day, randomly distributed throughout the light phase. The stress paradigm consisted of restriction in a 25 cm-high, 250 ml glass cylinder filled, up to a height of 5 mm with hand-hot water, whilst exposing them to bright light, as described by Behan et al. (2011). Control animals were left undisturbed in their home cages.

From the last stress session and weighing of dams on E17 up to postnatal day (P) 5, dams and their respective litters were left undisturbed. Pups were counted and litters were weighed at P5, P12 and P21, before weaning, and on P35 and P60, after weaning. At P25±3, pups were weaned and group-housed according to their genotype and condition. Female offspring were housed in groups of 3±1 at an inverted day-night-cycle (12 h:12 h; lights on from 7PM). Animals were allowed to grow up undisturbed, except for weekly cage changes, until the behavioural testing started at the age of 2 months. In brief, animals underwent behavioural screening, starting with elevated plus maze (EPM), followed by Porsolt swim test (PST), and 3-chamber sociability test (3-CST). Following behavioural testing (after 1 week of recovery) the corticosterone response to restraint stress was investigated in blood plasma. Subsequently, around P90, female offspring intended for further molecular investigation, were sacrificed using isoflurane, followed by quick decapitation. Brains were and immediately, carefully frozen in isopentane at -80°C.

Behavioural testing

All behavioural tests were performed in the dark (9AM - 7PM). Litters, smaller than 5 pups, were not considered (Tanaka, 1998). For all tests, mice were tracked using infrared light from below the respective apparatus and recorded from above with an infrared-sensitive camera. Recorded videos were analysed using the VideoMot2 tracking software (TSE Systems, Bad Homburg, Germany) or Ethovision Pro (Noldus, Wageningen, The Netherlands). In-between trials the respective apparatus was cleaned with Terralin liquid (Schülke, Norderstedt, Germany).

Elevated plus maze

The EPM, a plus-shaped apparatus made from opaque, black acrylic (TSE Systems, Inc., Bad Homburg, Germany), is semi-permeable to infrared light. It consists of two opposing open arms without walls (30 cm × 5 cm, with 0.5 cm wide boundaries elevated 0.2 cm) and two opposing closed arms (30 cm × 5 cm) enclosed by 15 cm high walls. The four arms meet in the centre forming a square (5 cm × 5 cm). The maze was 62.5 cm elevated from ground level. The test was performed under low light and each animal was placed in the centre facing an open arm (Post *et al.*, 2011; Gutknecht *et al.*, 2015). Each mouse was allowed to explore the maze for 5 min. From the recorded videos, time spent and distance moved in the open arms, the closed arms and the centre, as well as the number of entries into the different arms were analysed (Lister, 1987).

Porsolt swim test

The PST is a classic test for behavioural despair in rodents (Porsolt, Le Pichon and Jalfre, 1977). For the test, each mouse was placed in a 40 cm-tall acrylic glass cylinder of (19 cm diameter) filled with hand-hot water (31±1°C) for 10 min. Mice from one cage were tested in parallel in visually separated cylinders. The setup was illuminated from below with a light-box. In between trials, the water was renewed and the cylinders were cleaned with Terralin. From the recorded videos, distance moved was used as indicative parameters of mobility in the PST (Behan *et al.*, 2011).

3-chamber sociability test

The 3-CST test investigates social preference and anxiety (File, 1980). The apparatus was made of acrylic glass and consisted of 3 separate chambers, i.e. one middle chamber with two adjacent chambers of equal size connected by doorways of equal size for each chamber. Moreover, both side chambers contained a wire cage. Prior to testing, mice had 5 min of habituation in the apparatus and subsequently a conspecific was placed into one of the small wire cages. Each mouse was allowed 10 min of exploration. From the recorded videos, the time spent in the respective chambers was manually assessed.

Corticosterone response to restraint stress

For measuring the corticosterone response to acute stress exposure, we collected blood from the saphenous vein before (basal) and immediately after 20 min of restraint stress (stress). The stress procedure was performed, as described for PS. Blood samples were, subsequently, centrifuged and the plasma was then stored at -80°C. Plasma corticosterone concentrations were determined using a radioactive immunoassay (RIA) as described in more detail, previously (Van den Hove, Lauder, *et al.*, 2006).

Quantitative real-time PCR and bisulfite pyrosequencing

Both DNA and RNA were extracted from hippocampal tissue. For this purpose, frozen brains were semi-thawed on a cooling plate (-6°C) and the hippocampus was rapidly dissected using a stereo microscope (Olympus, Hamburg, Germany). Before extracting nucleic acids, the tissue of the left and right hippocampus was merged, powderised at -80°C and split into two homogenous portions to enable investigating RNA expression and epigenetic modifications on the DNA in the same animals. For the quantitative real-time PCR (qRT-PCR) 12 control offspring of each genotype, and 23 *5-Htt*$^{+/+}$ as well as 27 *5-Htt*$^{+/-}$ PS offspring were tested. For the Pyro-sequencing experiments 9-10 control offspring of each genotype, and 20 *5-Htt*$^{+/+}$ as well as *21 5-Htt*$^{+/-}$ PS offspring were tested.

RNA extraction and quantitative real-time PCR

Extraction of RNA was performed using a combination of the classic phenol-chloroform method and the column-based protocol of the commercial miRNeasy Mini kit (Qiagen) for fatty tissue. The frozen samples were homogenized in QIAzol lysis reagent with a precooled stainless steel bead (Qiagen), using the TissueLyzer (Qiagen) at 20 Hz and 4°C, for 60 s. Subsequently, homogenates were incubated for 5 min (RT), mixed with 60 µl of chloroform (Roth, Karlsruhe, Germany) and incubated for another 10 min on ice, before the aqueous phase was recovered by phase separation and mixed with the 1.5 volumes of ethanol (95-100%). Then samples were transferred to miRNeasy mini spin columns

and consecutively washed using the kit specific RWT and RPE buffer. To minimise the risk for gDNA contamination the samples were incubated with DNase (Qiagen) in-between RWT washes, according to the manufacturer's instructions. RNA was finally eluted in RNase free H_2O, provided with the kit and stored at -80°C. RNA quality was assessed before further processing using the Experion (Biorad, München, Germany) according to the manufacturer's instructions. Only samples with Experion RNA quality indicator (RQI) between 8.0 and 9.8 were considered for further analysis. RNA concentrations were determined on the Nanodrop (Thermo Fisher Scientific). Following extraction, reverse transcription was performed using the iScript kit (Biorad, Munich, Germany) with an initial input of 500 ng RNA. Copy DNA (cDNA) was diluted 1:5 using 1x TE and stored at -20°C until further use. In addition to our test samples, we also included five inter-run calibrator samples and three non-transcribed samples.

The qRT-PCR was performed according to the MIQE guidelines (Bustin *et al.*, 2009). *Mbp* was used as the target gene, while LIM and calponin homology domains 1 (*Limch1*) and rho GTPase activating protein 26 (*Arhgap 26*) were included as reference genes, as they were suggested as most stable by GeNorm. In addition to the GeNorm analysis, we performed a statistical analysis to validate that reference genes were stably expressed between groups. All Primers were designed using the NCBI Primer Blast tool, which is based on the Primer3 algorithm (Koressaar and Remm, 2007; Untergasser *et al.*, 2012). If applicable, all primer pairs were designed as intron-spanning to avoid amplification of gDNA contamination (Primers: **Table S 2**). Furthermore, three non-transcribed samples were included in every run, to detect possible gDNA contamination in case primer pairs were not intron-spanning. As mastermix, the SYBRSelect Master Mix (Thermo Fisher Scientific) was used and qRT-PCR reactions were performed in a 384-well format on the CFX384 Real-Time PCR Detection System (Bio-Rad, München, Germany). All samples and controls were run in triplicate and per target gene all samples were placed on one plate. Following amplification, melting curves, amplification curves, non-template controls and non-transcribed samples were assessed. A representative number of samples per target were also checked on agarose gels to determine the specificity of the primer pairs. Following these quality control steps the data was further analysed using LinReg (Ruijter *et al.*, 2009) to determine the PCR efficiency of every run. Only samples with efficiencies within 5% of the calculated mean PCR efficiency were taken into account. Moreover, we checked triplicates for consistency and all samples that lay outside an interval of 0.5 Cq per triplicate were excluded from further analysis. Finally, data was normalised and corrected for inter-run differences between plates and repeat plates using the qbase$^+$ software (Biogazelle, Zwijnaarde, Belgium).

S 2 Table Primer pairs used for the quantitative real-time investigation of mRNA expression of myelin basic protein (Mbp) in mice. Primer pairs are covering, Mbp isoforms with (1/2) and without (1/3) exon 2.

Name	modifications	Sequence	Product size [bp]
Mmu_qRT_Mbp_ex1/3_F		ACAGAGACACGGGCATCCT	90
Mmu_qRT_Mbp_ex1/3_R		TGTGTGAGTCCTTGCCAGAG	
Mmu_qRT_Mbp_ex1/2_F		ACAGAGACACGGGCATCCT	89
Mmu_qRT_Mbp_ex1/2_R		CCAGGGTACCTTGCCAGAG	
Mmu_qRT_Limch1_1_F		GTCGGAAGATGTGAAGCCCA	110
Mmu_qRT_Limch1_1_R		AGGAGCACAGTTTCTTCCCG	
Mmu_qRT_Arhgap26_2_F		AGACGAGTCCGTTACCCTCA	119
Mmu_qRT_Arhgap26_2_R		ACAACGCCTGCATGGTGATA	

DNA extraction and bisulfite-conversion-based pyrosequencing

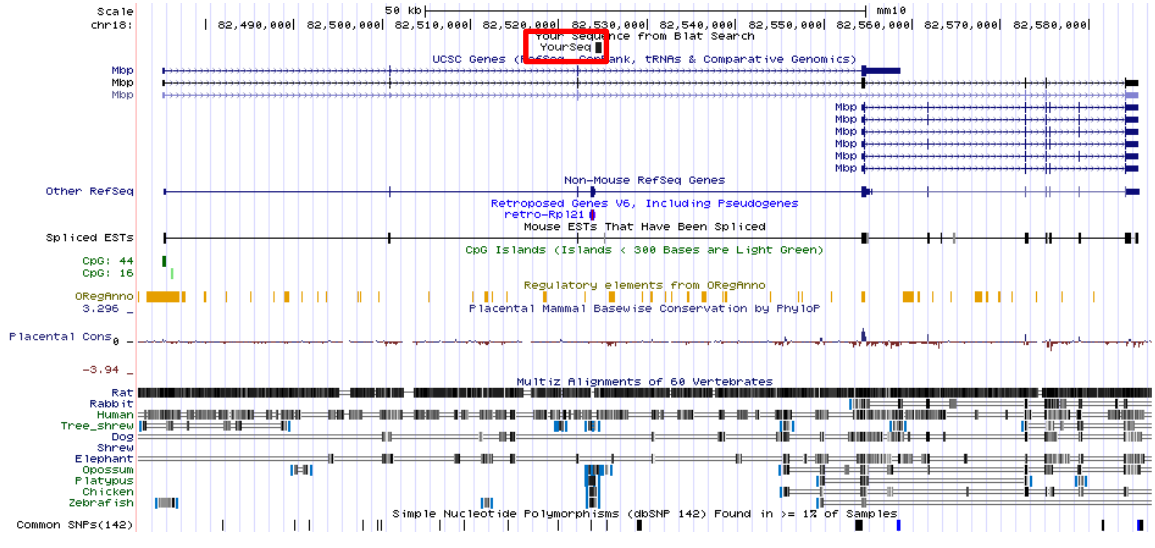
Extraction of gDNA was performed using a standard phenol/chloroform/isoamyl alcohol extraction protocol as published elsewhere (Schraut *et al.*, 2014). In brief, frozen samples were homogenized, consecutively incubated with proteinase K and RNase A and subsequently DNA was isolated, using phenol/chloroform/isoamyl alcohol solution (25:24:1). Following isolation, DNA was precipitated and washed with ice-cold ethanol. Finally, the pellet was air-dried and resuspended in 1x TE. DNA was stored at -80°C for further use. DNA concentrations were determined using the Nanodrop (Thermo Fisher Scientific). Following extraction, bisulfite-conversion was performed using the EpiTect Fast Bisulfite Conversion kit (Qiagen) according to the manufacturer's instructions. 800 ng of DNA were diluted in 20 µl RNase-free water, 85 µl bisulfite solution as well as 35 µl DNA protect buffer were added to a total reaction volume of 140 µl. Following the conversion and subsequent washing steps, biDNA was aliquoted and stored at -20°C for further use. Subsequent bisulfite-conversion-based pyrosequencing was performed as described earlier, but with only two replicates per sample. As for the human study a difference of 5% were accepted and the analysis was performed using the mean of both replicates. Primers are depicted in **Table S 3**.

S 3 Table Primer pairs used for the bisulfite-conversion-based pyrosequencing analysis of a myelin basic protein (Mbp) in mice.

Name	Modifications	Sequence	Product size [bp]
Pre-PCR primer			
PP-Mbp-C-F		TTTGATTGAAGGTAGAATAATGTAGAAG	193
PP-Mbp-C-Rb	Biotin	ACTCACCAACACCAATATAAATTATACA	
Sequencing primer			
Seq-Mbp-C-CG10		AGGTAGAATAATGTAGAAGTT	
Seq-Mbp-C-CG11-13		TTTTTGAGGTTTGGGAT	
Seq-Mbp-C-CG14-15		GTGGTATGAAGGTATTTTAG	

The primer pairs, used in the animal part of this study, have been previously published (Schraut *et al.*, 2014)

Primer location in the UCSC Genome Browser on Mouse Dec. 2011 (GRCm38/mm10) Assembly:



Your Seq:
chr18:82,524,190-82,524,827

CGGAGTCTGCTGAGACCCTTTACTTGTGTTTTCTAAGTATGTCCATAGAAGTAACACACATCTATATACACAGAGGA
GTATCGGGTACCAAAGGGAAGGCATAATATATACGCTCAGATTTGCGTTTTCTTTAAGCAAGTGTCATCTTTAGTAT
TAGTTATGATATTTTGTTATGCTAGAGGTTGAATTCAGGAGTTGAACACATGTTTAAGTGACTGCTCTACCACCGAG
CTGTGGCACTAGTCCCAATTAACCGATTTTTAATTTACTTATAATGATAGCAGCCCTTGTGTAACATTGTGCATATGC
GTATATGTGTGTGTACACACATACAAAATGTATGTAATGCACAAACCATGTCTTACAAGAGGCCTCTGTGAGCATCC
TCCTCTGATAGTATAAACACTCTGATTCCTGAAGCTGTCTCCCCTGAAGCCACCTTCA**CCCTGACTGA**AGGTAGAA
CAATGCAGAAGTT>>>GCG$_{8}$GGTCTCTCCACAACATGGTGGCTAGTGCTTG**TTCCTGAGGTCTGGGAC>>>**ACAGA
TATACTCG$_{9}$CG$_{10}$CCCG$_{11}$TGGCATGAAGGCACCTCAG>>>>AGCTCTTG**CAGTGT**AGACG$_{12}$CAGTAGGATCCG$_{13}$AGT
CAC CCTCACACA**TGCACAACTCATACTGGTGCTGGTGAGT**

- SOX10-binding sites
- CG: analysed CpGsites 8-13 according to (Schraut *et al.*, 2014)
- **PrePCR primer**
- **Seq primer>>>**

Association of adult behaviour and *Mbp* expression and methylation

S 4 Table Multiple linear regression of myelin basic protein (*Mbp*) methylation (CpGs 8-13) and expression (isoforms with and without exon 2) with several relevant behavioural parameters, i.e. anxiety-related elevated plus maze, 3-chamber sociability test, depression-related Porsolt swim test and corticosterone, before and after restraint stress, dependent on serotonin transporter genotype and prenatal stress condition.

Test		Stat	CpG8	CpG9	CpG10	CpG11	CpG12	CpG13	Mbp+Ex2	Mbp-Ex2
Mbp expression	Mbp + Exon 2	Est	0.011	0.002	-0.003	-0.003	0.002	0.002		
		t	0.686	0.577	-0.705	-1.314	0.527	0.681		
		p	0.496	0.566	0.484	0.195	0.600	0.498		
	Mbp - Exon 2	Est	0.008	0.001	-0.002	0.000	0.005	0.001		
		t	0.517	0.490	-0.399	-0.136	1.450	0.416		
		p	0.607	0.626	0.692	0.893	0.153	0.679		
elevated plus maze	Time -open arms	Est	-72641.6	-143.66	-25462.9	-27211.2	-22619.0	-37062.4	-405392.7	-388263.0
		t	-1.091	-0.011	-1.151	-1.767	-1.567	-1.858	-0.808	-0.813
		p	0.281	0.991	0.256	0.084	0.124	0.070	0.422	0.420
	Distance - open arms	Est	-148.37	-7.66	-86.46	-87.33	-79.83	-163.66	20.41	-441.46
		t	-0.752	-0.188	-1.295	-1.752	-1.799	-2.769	0.012	-0.264
		p	0.456	0.852	0.202	0.086	0.078	**0.008**	0.991	0.792
	Visit -open arms	Est	0.17	0.61	-1.32	-1.33	-1.65	-3.34	-24.34	0.08
		t	0.027	0.559	-0.647	-0.912	-1.332	-1.866	-0.520	0.002
		p	0.979	0.579	0.521	0.367	0.189	0.068	0.605	0.999
3-Chamber sociability test	Time -target	Est	-83.99	-0.61	1.70	20.78	38.45	47.18	262.38	324.69
		t	-1.170	-0.045	0.076	1.287	2.511	2.184	0.458	0.583
		p	0.248	0.964	0.939	0.204	**0.015**	**0.034**	0.649	0.562
Porsolt swim test	Distance	Est	801.38	58.91	199.13	36.99	-1.99	25.25	3886.02	-1243.41
		t	1.040	0.377	0.760	0.203	-0.012	0.101	0.581	-0.186
		p	0.303	0.708	0.451	0.840	0.990	0.920	0.563	0.853
Corticosterone	Baseline	Est	-30.39	34.43	-49.76	39.54	16.47	11.60	207.19	385.55
		t	-0.245	1.533	-1.322	1.347	0.604	0.305	0.218	0.418
		p	0.808	0.132	0.193	0.184	0.549	0.761	0.828	0.677
	Stress	Est	81.06	9.79	23.25	40.33	15.77	27.95	249.11	470.07
		t	0.732	0.439	0.664	1.619	0.682	0.827	0.299	0.588
		p	0.468	0.663	0.510	0.112	0.498	0.412	0.766	0.559
	Diff	Est	111.45	-24.65	73.01	0.80	-0.70	16.35	41.93	84.53
		t	0.890	-1.012	1.839	0.027	-0.026	0.421	0.043	0.089
		p	0.378	0.317	0.072	0.978	0.979	0.675	0.966	0.929

Immunohistological and histological investigation of myelin distribution and cell-type composition

For the immunohistological and histological investigation a second cohort of animals was created as previously described. Following weaning at P25±3, animals were housed in groups of 2-7 at a 14 h/10 h light-dark cycle with lights on at 7AM - 9PM. Animals were allowed to grow up undisturbed. At P90±3 the animals were sacrificed using isoflurane followed by cervical dislocation and transcardial perfusion with 4% paraformaldehyde (PFA). Following perfusion, brains were post-fixed for 24 h and immersed in 20% sucrose before they were frozen in precooled isopentane and stored at -80°C.

Immunohistochemical approaches

For our immunohistochemical analysis, the PFA-fixed brains were sectioned from Bregma 1.33 mm up to -3.63 mm using a cryostat (Leica Biosystems, Nussloch, Germany) at a temperature of -20°C. Brains were cut in 30 µm thick sections and in total 6 series were mounted on Histobond+ slides (Marienfeld Superior, Lauda-Königshofen, Germany).

APC and MBP staining

To determine the number of OLs as well as the levels of MBP, immunohistochemical stainings were employed. The stainings were performed in two distinct series using an indirect 3,3'-diaminobenzidine (DAB) staining. For both immunohistochemical protocols, inactivation of endogenous peroxidases was performed using 0.6% hydrogen peroxide in 1x PBS for 30 min at RT, followed by antigen-retrieval at 85°C for 20 min, using 1x DAKO antigen retrieval solution (DAKO, Agilent Technologies, Santa Clara, CA, US) for the adenomatous polyposis coli protein (APC), and 10 mM citric acid at pH 6.0 for the MBP staining. Next, the samples were incubated for 90 min at RT with blocking solution, using 5% normal goat serum (NGS) [5% NGS, 0.25% triton X-100, 2% BSA in 1x PBS] and 5% normal donkey serum (NDS) [5% NDS, 0.25% triton X-100, 2% BSA in 1xPBS] for the APC and MBP staining, respectively. After blocking, the primary antibody was directly added to the respective slides. For the APC staining, we used a mouse anti-APC monoclonal antibody (Merck Milliepore, Darmstadt, Germany) at a dilution of 1:200 in NGS blocking solution for 48 h at 4°C, while for the MBP staining we used a rabbit polyclonal anti-MBP antibody (ab40390, Abcam, Cambridge, UK) at a dilution of 1:200 in NDS-blocking solution. Every step, up until blocking, was followed by three washes in 1x PBS à 5 min at RT.

Following incubation with the primary antibody, the tissue was washed for 3x 10 min in 1x PBS at RT. All subsequent washing steps were performed accordingly, unless stated otherwise. Next, a biotinylated goat anti-mouse antibody (VectorLaboratories, Burlingame, US) at a concentration of 1:400 in 2% NGS blocking solution, or donkey anti-rabbit antibody (VectorLaboratories, Burlingame, US), at a concentration of 1:250 in 2% NDS blocking solution, were added to the APC and MBP slides, respectively, and incubated for 90 min at RT. Subsequently, sections were incubated with the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, US) diluted at 1:100 in 1x PBS for 90 min at RT. Finally, sections were stained using a DAB solution for 10 min to obtain a colour reaction. After rinsing in 1xPBS, sections were either dehydrated and cover-slipped with Depex (Klini-path, The Netherlands; MBP staining) or counterstained with cresyl violet (4 min 0.1% cresyl violet, followed by

30 s in 70%, 96% and 100% ethanol, 2 min in 1:1 mixture of xylene and isopropanol and 4 min in xylene), dehydrated and mounted using Vitro Clud (NOVOGLAS , Langenbrinck, Germany; APC staining).

Nissl staining

To identify the total number of cells in the hippocampus, we performed a Nissl staining. Firstly, the mounted sections were taken out of the -80°C freezer and left to dry at RT for 30 min. Secondly, the slides were incubated for 20 min in a 1% acetic acid solution, followed by 20 min incubation in 75% ethanol with 0.025% Triton-X-100 and another 20 min incubation in a 1% acetic acid solution. Finally, the slides were placed in 0.1% cresyl violet for 15 min followed by 3 times washing for 1 min in acetic acid buffer. Before cover slipping with Depex (Klini- path, The Netherlands), the tissue was dehydrated by incubating the slides in 100% ethanol for 1 min, isopropanol for 2x 5 min, and xylene for 2x 5 min.

Stereological analysis

To analyse the number of OLs and the total number of cells, we used a stereological computerised microscopy system and the Stereo Investigator software (MBF Biosciences, Williston, VT, US). We counted APC immunoreactive (ir) cells as well as the total number of cells (Nissl) using design-based stereology. Briefly, within the hippocampus: granular cell layer (GCL), dentate gyrus (DG), cornu ammonis (CA) and oriens layer (orL), were delineated at a 10x magnification on live microscopic images, displayed on a monitor. The borders were delineated between Bregma -1 and -3.4 as this area reflects the region we dissected for the molecular analyses. We investigated every second section. Missing sections were skipped. Subsequently, cells were counted at a 100x magnification with oil, using the optical fractionator method (Schmitz and Hof, 2005). Counting frames (40 x 40) were created by the software and positioned at the intersections of a virtual grid (150 x 150) that had been placed over the image. For our study, we counted every second counting frame moving stepwise in the x and y- direction. The optical dissector height, along the z-axis, was determined and resulted in a sampling fraction height of 7 µm for the Nissl staining and 14 µm for the APC staining. Cells were counted if they showed clear staining and were in focus within the counting area. The relative cell density was calculated by dividing n(counted cells)/area(counted frame parcells) of the region of interest.

Densimetrical analysis

MBPir was evaluated by means of a semi-quantitative analysis. The acquisition of images was done with an Olympus Provis AX70 microscopy system with the Cell-P imaging software (Olympus, Tokyo, Japan), connected to a digital camera (F-View; Olympus, Tokyo, Japan). Subsequently, the NIH ImageJ software (http://rsb.info.nih.gov/ij/) was used for the analysis of area coverage. Regions of interest (ROIs) were defined in the images of the Schaffer collaterals, fornix and hilus at Bregma -2.03 mm and -3.07 mm. Each image was set to an automatic threshold value and blood vessels as well as artefacts were excluded from the analysis. The relative area coverage by MBPir was then calculated by dividing the measured area covered by the total area of the ROI. The whole analysis was conducted by an investigator blind to the experimental conditions.

Statistical analysis

All data were collected in Microsoft Excel (Microsoft, Redmond, WA, US) and further exported and analysed in R (version 3.4.2; The R Foundation, Vienna, Austria) and RStudio (version 1.0.143; The Foundation for Open Access Statistics, Boston, MA, US). Extreme outliers were excluded from the analysis and log transformation was performed to address skewed data.

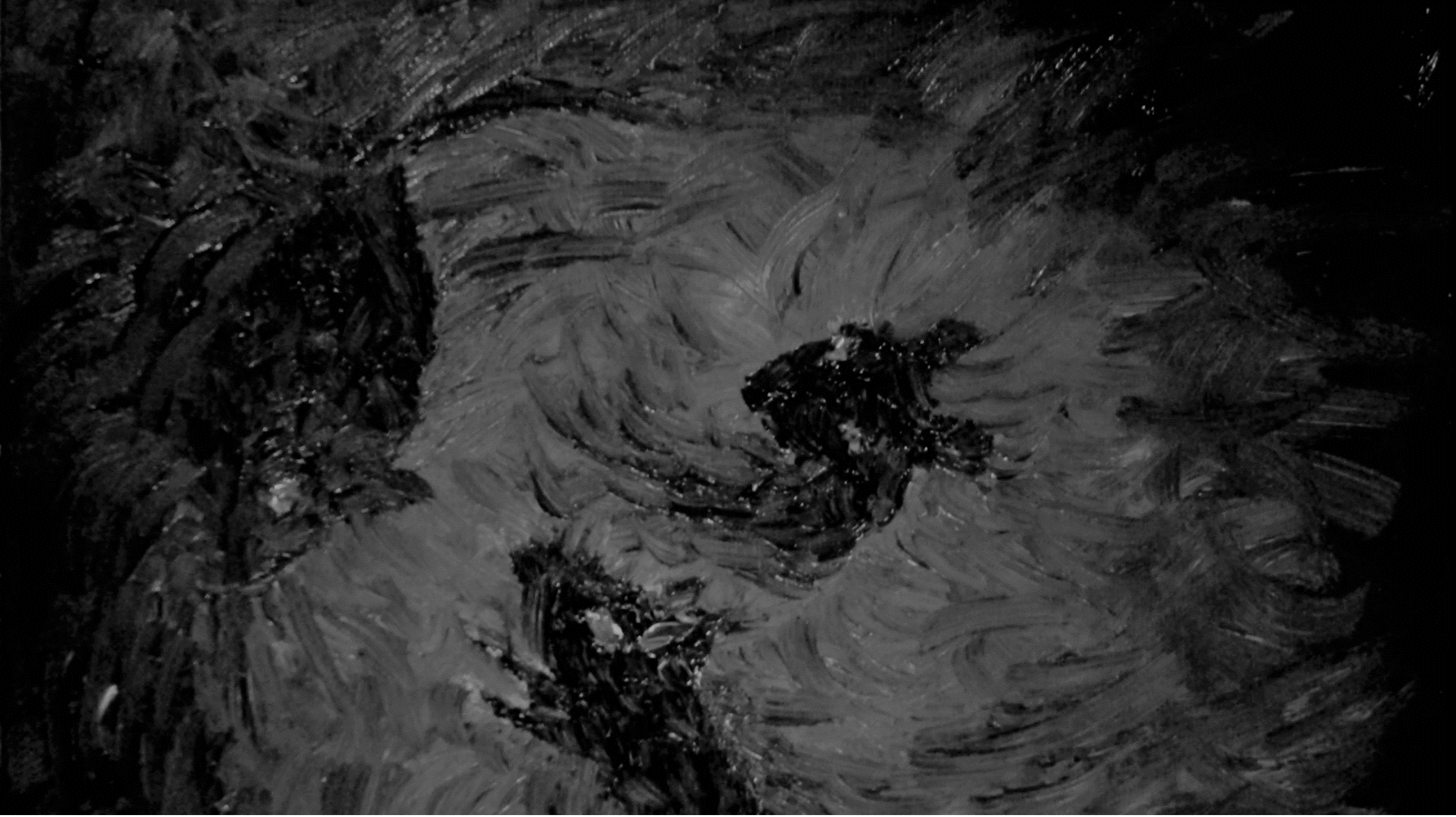
22 distinct histological measures (APC and Nissl cell density and ratio in GCL, DG, CA and the orLs as well as MBP area coverage in fornix, hilus and Schaffer collaterals) were set as dependent variables and an interaction of condition (prenatal stress, no stress) and *5-Htt* genotype (wildtype, heterozygous) as predictors. Next to the general approach a hierarchical linear model was applied and clustered per litter as well as corrected for litter size, when the increase in dam weight [(E17-E13)/E17] or its interaction with *5-Htt* genotype was used as predictor.

Results

Concerning the histological parameters, we found no association of maternal condition or relative body-weight gain in any of the histological measures. Interestingly, thought, the *5-Htt* genotype of offspring was found to be associated with the area covered by MBPir in the Fornix at Bregma -3.07 (Est = -9.916, t = -2.14, p = 0.043), with *5-Htt*$^{+/-}$ animals having a comparably lower coverage of the area with MBP immunoreactivity.

Conclusion

Next to DNA methylation and RNA expression, immunohistological investigation was undertaken to further investigate the origin of the observed molecular alterations. To this end, both cell-type composition and MBPir was investigated and revealed that none of the investigated measures were affected by PS or relative maternal weight gain. Interestingly, though, abundance of MBP in the fornix at Bregma -3.07 mm was associated with offspring *5-Htt* genotype, with an overall lower MBP immunoreactivity in the fornix of *5-Htt*$^{+/-}$ offspring. Even though this effect was only subtle, this observation might suggest a general alteration in myelination, dependent on the 5-Htt genotype, which might explain the genotype-specific epigenetic programming.



General discussion

Anxiety, aggression and related disorders are a major social and economic burden in today's society, with symptoms, such as the excessive worry in generalised anxiety disorder (GAD) or panic attacks, and inflicting harm on others or self, that impair patients in everyday life and are putting a strain on communities and families (Penden, McGee and Krug, 2000; World Health Organization, 2002; Kessler and Greenberg, 2012). For both patients and society, it is therefore extremely relevant to extend the current understanding of the underlying brain circuitries and molecular mechanisms of overshooting fear and anxiety, antisocial behaviour and other related traits in order to promote more efficient, targeted, and individual treatment options. In this respect, the approaches used in the current thesis were aimed to answer, at least in part, some of the most relevant questions regarding the involvement of the serotonin (5-hydroxytryptamine, 5-HT) system in aetiology and manifestation of such disorders. Taken together, research reported in the previous chapters supports a role of 5-HT in modulating the effects of early adversity through epigenetic programming of the development of the brain and, consequently, associated behaviours.

As discussed in **chapter 2**, it seems that, with regard to abnormal, aggression-related behaviours, the 5-HT system can serve as a distinctive regulator. Alterations in the regulation of the 5-HT system and concomitant changes in 5-HT signalling have been identified as critical factors, underlying the manifestation of aberrant behaviours in humans, non-human primates, and rodents. Animal models of early-life stress have emphasised the complexity of effects and interactions that occur in response to disturbances throughout development. Next to a wide range of behavioural changes in affected animals, distinctive changes in the 5-HT system have been observed at multiple levels of 5-HT regulation and signalling. Critical players in this respect are e.g. tryptophan hydroxylase 2 (TPH2) (K. L. Gardner *et al.*, 2009b; Miyagawa *et al.*, 2011; Hiroi *et al.*, 2016), the rate limiting enzyme in 5-HT synthesis, 5-HT transporter (5-HTT) (Katherine L. Gardner *et al.*, 2009a; Kinnally *et al.*, 2010; Kinnally, Capitanio and Leibel, 2010), various 5-HT receptors (Van den Hove *et al.*, 2006; Holloway *et al.*, 2013) that are involved in regulating and mediating the effects of 5-HT signalling, and monoamine oxidase A (MAO-A) (Márquez *et al.*, 2013; Wong *et al.*, 2015), responsible for the degradation of 5-HT, following reuptake into the cell. Moreover, in some of these studies, the observed alterations in the 5-HT system were linked to alterations in epigenetic marks at 5-HT system-related genes themselves, suggesting an intricate epigenetic regulation of 5-HT-related changes in the context of early adversity (Kinnally, Capitanio and Leibel, 2010; Márquez *et al.*, 2013). Furthermore, 5-HT signalling was shown to be involved in epigenetic programming of other systems by early adversity. For example, in rodents, early-life programming of the HPA-axis via altered 5-HT signalling and turnover was suggested (Smythe, Rowe and Meaney, 1994; Meaney *et al.*, 2000; Laplante, Diorio and Meaney, 2002).

To investigate the suggested role of 5-HT in, and identify potential targets of 5-HT-linked, epigenetic regulation, by which early adversity programs adult emotion-related behavioural phenotypes, **chapter 3** addressed the consequences of TPH2 deficiency on the molecular make-up of the brain and associated behaviours, in the context of early adversity. To this end, litters of TPH2-deficient dams, depleted of brain 5-HT, were exposed to neonatal maternal separation (MS), and male offspring,

carrying the null mutation (-/-), as well as heterozygous (+/-) and wildtype (WT) littermates, were examined in several anxiety and sociability-related tests, in adulthood. Besides the behavioural screening, whole genome analysis of DNA methylation and RNA expression was performed in the same animals, in order to allow for an associative identification of altered transcriptional regulation in mice with distinct early experiences and 5-HT system functionality. In mice, fully depleted of brain 5-HT, a strong shift towards offensive and activity-oriented behavioural responses was observed in either task, compared to WT offspring of the control group, independent of early adversity. Furthermore, *Tph2*$^{+/-}$ knockout mice were found to display ambiguous behaviours comparable, to WT or *Tph2*$^{-/-}$ knockout animals, for activity- and anxiety-related tasks or social tasks, respectively. Moreover, it seems, that MS washed out the observed differences between WT and *Tph2*-deficient offspring, dependent on the behavioural context (i.e. task interrogated) in adulthood. These observations were subtle but might suggest an interplay between developmental adaptions in response to early adversity, a general (chronic) deficiency of 5-HT throughout development, and the acute lack of 5-HT during behavioural testing in adulthood. Furthermore, it is possible that the maternal stress susceptibility exerts an additional modulatory effect on offspring behaviour (Meaney, 2001).

On the molecular level, with regard to RNA expression and DNA methylation in the amygdala, we observed that the levels of RNA expression were dependent on both *Tph2* genotype and its interaction with MS. For example, we observed a threefold higher number of differentially expressed genes (DEGs) when assessing *Tph2* genotype x MS effects in *Tph2*$^{+/-}$ versus WT offspring when compared to assessing *Tph2*$^{-/-}$ versus WT animals. At the same time, the number of differentially methylated loci (DMLs) for these two comparisons was similar. The number of overlapping DEGs and DML-associated genes was notably low. This observation is most likely to be accounted for by a more complex mechanism of regulation, as for example observed by (Zhang *et al.*, 2013). Nevertheless, when investigating those genes that are showing both differential expression and methylation, we found that cholecystokinin (*Cck*) expression correlated significantly not only with DNA methylation, but also with behaviour. Similarly, *Cck* gene methylation was observed to correlate with behaviour. CCK is one of the most abundant neuropeptides (Innis *et al.*, 1979; Crawley, 1985; Moran and Schwartz, 1994) and expressed, amongst others, in several amygdalar subnuclei (Asan, Steinke and Lesch, 2013). Next to CCK signalling as a potential effector, we identified two distinct pathways that might be involved in explaining the observed, *Tph2* genotype-dependent behavioural profiles. When assessing *Tph2* genotype x MS effects in *Tph2*$^{+/-}$ versus WT offspring “cytokine signalling” was identified as significantly enriched both, at the level of gene expression and DNA methylation. Cytokine signalling has been shown to modulate emotional behaviour by interacting with the 5-HT system (Lowry *et al.*, 2007). When examining *Tph2* genotype x MS effects in *Tph2*$^{-/-}$ versus WT offspring, “exercise induced circadian regulation” was identified as an enriched pathway, which provides a potential explanation for the observed alterations in activity-related behaviours and concomitant physiological measures.

The role of 5-HT in developmental programming was further addressed in **chapter 4**, where adult WT and 5-HTT-deficient offspring of C57BL/6 dams that were exposed to a restraint stress paradigm during pregnancy (prenatal stress; PS) were investigated on the behavioural level for anxiety and social behaviours as well as at the level of hippocampal epigenetic marks and mRNA expression. Generally, PS reduced sociability, in particular in 5-Htt$^{+/-}$ offspring. This effect was subject to a high inter-individual

variability, with a substantial number of PS offspring showing normal social behaviour and an almost equal number of animals showing decreased social behaviour following PS. In accordance with previous findings (Jakob *et al.*, 2014), genome-wide hippocampal profiling of gene expression and H3K4me3 enrichment revealed that differential susceptibility to PS was associated with a distinct behavioural profile, as well as numerous molecular changes, most of which were dependent on the *5-Htt* genotype. Similar to observations in **chapter 3**, we found a pronounced difference between genotypes with regard to the number of regulated DEGs and a more moderate difference in the number of differentially enriched H3K4me3 loci (DELs). Similar to observations in DNA methylation, the number of overlapping DEGs and DELs was relatively low, for any comparison. This finding is in line with the findings described in a previous *5-Htt* x PS study, undertaken within our group (**Supplementary chapter** (Schraut *et al.*, 2014)), and, more general, with the current understanding of epigenetic regulation in eukaryotic cells. In WT offspring, no DELs showed overlap with DEGs, which is in line with the overall, relatively lower degree of PS-induced gene expression changes in both socially normal and socially anxious WT offspring. In contrast, in 5-HTT-deficient offspring, a small fraction of DEL-associated genes also reflected DEGs. This, on the one hand, supports the hypothesis that 5-HT is involved in programming by PS and, on the other hand, strongly suggests further levels of epigenetic regulation, directing gene expression. Of the genes affected in both, expression and H3K4me3 enrichment, oligodendrocyte transcription factor 1 (*Olig1*) represents a most interesting candidate, as next to *Olig1*, the expression of several other myelination-associated genes, as well as some enriched pathways, involved in oligodendrocyte formation and development, were found to be altered in *5-Htt*$^{+/-}$ offspring that showed normal social behaviour following PS. This is in accordance with numerous findings, linking developmental stress and the 5-HT system to myelin-related factors (e.g.(Schraut *et al.*, 2014; Bennett *et al.*, 2015)). Next to these myelination-related changes, “exercise-induced circadian regulation” was identified in this study to be altered exclusively in *5-Htt*$^{+/-}$ offspring, dependent on differential susceptibility.

In conclusion, both studies undertaken with the aim of identifying the effects of brain 5-HT signalling throughout development and in interaction with exposure to early adverse conditions, were able to show notable evidence on the role of 5-HT in mediating the effects of early-life stress exposure. One pathway that had been found to be specifically altered in a 5-HT-dependent manner in both screening studies was the “exercise-induced circadian rhythm” pathway, which might represent a likely target for future research on 5-HT-related early-life programming.

In parallel to these more exploratory approaches, we also aimed for a dissection of neural networks, involved in mediating the effects of developmentally programmed 5-HT system (dys)function. In **chapter 5**, we assessed the effects of TPH2 deficiency in interaction with neonatal MS on c-Fos abundance in several, relevant brain structures following behavioural testing. *Tph2*$^{-/-}$ offspring and their heterozygous and WT littermates were tested in distinct anxiety-related tests in adulthood and, subsequently, sacrificed for immunohistological investigation of neural activation (c-Fos) in response to the test. Taken together, our findings in this study suggested that, dependent on the availability of brain 5-HT, MS promotes active coping in response to aversive stimuli, dependent on the context. This is supported by observations recently published in (Waider *et al.*, 2017) and partially in line with findings previously described in **chapter 3**. Furthermore, the observed increase in neural activity was distinctive

for the different behavioural tests, as well as across *Tph2* genotypes, in several of the investigated brain regions. The neural activation of the lateral (La) and basolateral (BL) amygdala seemed to be intriguingly specific for the employed anxiety test with a general activation of the La following the DLB test and an MS-dependent activation of the La following OF testing. The La is receiving and processing most of the sensory input (Asan, Steinke and Lesch, 2013). Therefore, MS-dependent activation in OF-tested mice might represent a relevant mediator of panic-related behaviours, dependent on early life experiences in TPH2-deficient offspring. The BL, in turn showed the highest activation following the OF test, while following the DLB test, BL activation was only observed in *Tph2*$^{+/-}$ animals. In a previous study, this higher activation in *Tph2*$^{+/-}$ animals was suggested to be a compensatory effect (Waider *et al.*, 2017). The test-dependent activation might be accounted for by the functional profile of the BL, which has been suggested to integrate information to evaluate environmental stimuli (Asan, Steinke and Lesch, 2013). For example, corticotrophin-releasing hormone (CRH) was suggested to facilitate BL activation (Paul *et al.*, 2014). This could explain the proportionally higher activation of the BL upon exposure to the more aversive (i.e. stressful) and inescapable OF test in comparison to the less aversive DLB test. The central amygdala (Ce) is the major output station of the amygdala and was activated by either test in a similar fashion. Moreover, the activation was independent of *Tph2* genotype and MS. The observed lack in differential neural activation in the Ce might be accounted for by a lack of resolution. In this study we did not identify neuronal subtypes, rendering us ignorant of cell-specific, neural activation. The Ce comprises a network of highly complex and specific interneurons responsible for a multitude of distinct behaviours (Asan, Steinke and Lesch, 2013), and, therefore, the identification of cellular subpopulations might be necessary to relate behavioural profiles to neural activation. Next to the differential activation of several amygdala subnuclei, we found a distinctive neural activation of the paraventricular nucleus (PVN). DLB testing evoked neural activation of the PVN only in *Tph2*$^{+/+}$ and *Tph2*$^{+/-}$ animals, however, independent of MS, while OF testing activated the PVN, independent of *Tph2* genotype and MS. This might be indicative for a more complex nature of the experienced stress in DLB testing and might suggest the integration of additional input from forebrain regions and brain stem structures such as the raphe nuclei (Van de Kar, 1997; Herman *et al.*, 2005). A potential link of 5-HT raphe neurons and PVN activation finds further support in diverse studies, linking the PVN to 5-HT signalling (Gibbs and Vale, 1983; Liposits, Phelix and Paull, 1987; Herman and Cullinan, 1997; Van de Kar, 1997; Herman *et al.*, 2005). In light of the, in **chapter 3** discussed, reciprocal effects of 5-HT and stress as well as, as reported in contemporary research elsewhere, we made the amygdala out as an important hub of early adversity-induced programming. In support of this notion, for example, Donner and colleagues observed that priming the amygdala with a CRH receptor (CRHR) agonist resulted in increased expression of *Tph2* mRNA in the dorsal raphe (DR) (Donner, Johnson, *et al.*, 2012). Similarly, male rats that received chronic corticosterone via the drinking water showed an upregulation of TPH2 protein levels in the light phase, abolishing the diurnal expression pattern of this enzyme (Donner, Montoya, *et al.*, 2012). Further investigation revealed a stress-dependent increase in TPH2 activity in the dorsal and caudal DR, following acoustic startle. Pharmacological CRHR2 blockade prevented this increase of TPH2 activity, whereas CRHR1 blockade enhanced TPH2 activity (Donner *et al.*, 2016). This is in line with findings that suggest a close inter-regulatory action of a specific subset of 5-HT DR neurons and CRH-expressing neurons of the Ce and through such of 5-HT and CRH (Asan, Steinke and Lesch, 2013). Interestingly, this intricate 5-HT/CRH network seems to be crucially involved in the regulation of panic and stress-induced hyperactivity and is supposed to be

counteracted by projections from the DR to the amygdala promoting anxiety-related responses such as freezing (reviewed in (Paul *et al.*, 2014)). Notably, the concept suggested in the current study, still suffers from a lack in differentiating between either chronic, long-term programming effects in response to adverse early-life conditions (known to change 5-HT signalling) or actual (acute) 5-HT deficiency. Of particular interest might also be the role of *Cck*-positive interneurons that can be found in all subnuclei of the amygdala and that had been shown to receive 5-HT input (Asan, Steinke and Lesch, 2013).

In **chapter 6,** we investigated the effects of early adversity on myelin-associated parameters in the context of 5-HTT deficiency. In this respect, we aimed to validate the programming of myelin basic protein (*Mbp*) by PS in human infants and in another mouse study, following up on our molecular findings from **chapter 4** and the **supplementary chapter**. Overall, we found that in humans as well as in mice maternal stress during pregnancy altered the DNA methylation at the *MBP* gene near one transcription start site. This modification occurred only in female infants, in humans, and was dependent on the *5-Htt* genotype, in mice. Histological analysis of cell-type composition showed furthermore, that the observed signal was not due to an altered cell-type composition in the investigated tissue. Furthermore, MBP immunoreactivity was altered in a genotype-dependent manner. Taken together, these observations and the fact that maternal stress affected *MBP* methylation in umbilical cord blood in a similar way as in murine, hippocampal tissue, suggest an epigenetic priming rather than direct influence of early adversity, supporting the previously discussed multi-layered nature of epigenetic regulation, which, in this way, allows the implementation of early and later experiences into an individual's interactions with its environment.

All in all, this thesis adds evidence concerning the role of 5-HT in light of the "developmental origin of health and disease" (DOHAD) concept (Barker, 2007), at the behavioural, brain circuitry and molecular, i.e. protein, RNA and DNA level.

However, next to shedding more light on the mechanisms involved, several limitations of the applied approaches become apparent. In terms of simple numbers: for example, the restricted number of animals used, the low amount of specific tissue available, and the high costs of whole genome approaches cannot be overlooked. Studying gene-by-environment interactions are a logistical challenge per se, as due to the highly complex experimental design, the number of experimental groups is notably higher when compared to single factor designs. This is often associated with a decrease in group sizes, as the manageable and feasible number of animals is strictly limited due to e.g. breeding programs, simultaneous behavioural testing and available financial resources. Furthermore, as the expected transcriptomic and epigenomic changes are rather subtle, genome-wide analyses, as performed in this thesis, usually yield few or no marks that survive correction for multiple testing.

Moreover, to which degree complex factors, such as early adversity and altered 5-HT system signalling and their interaction are adequately captured in an experimental setup can be questioned. This holds particularly true when considering the fact that their impact on epigenetic changes is highly complex. Moreover, the observable factors of epigenetic regulation, i.e. histone modifications, DNA (hydroxy)methylation and DNA binding factors are not stand-alone regulators, but, rather, exert their

effects in concert, creating a specific chromatin state. Thus, through the dynamic regulation of chromatin, properties of the associated transcription start sites and, consequently, gene expression can be changed (Jenuwein and Allis, 2001; Narlikar, Fan and Kingston, 2002; Barski *et al.*, 2007; Garske *et al.*, 2010; Kratz *et al.*, 2010; Fuchs *et al.*, 2011). Furthermore, epigenetic changes are not only dependent on early-life environment, but accumulate additional layers throughout life, dependent on the molecular make-up, sex, tissue-type and many other factors, even including stochastic epigenetic mutations (Petronis, 2010). Some of these imprints might, in turn, regulate the likelihood of future epigenetic programming at affected sites. And while some of the superposed marks might be removed or replaced with time, others will be more permanent. Therefore, it is becoming more and more apparent that recent approaches addressing only 1-2 levels of epigenetic regulation are far from optimal. Hence, one of the main future challenges in the field of neuroepigenetics is to generate tools that capture epigenetic and transcriptional regulation at a time- and dimension-integrated systems level.

Another strong limitation is the aspect of specificity, as epigenetic programming, but also brain circuitry signalling, is highly cell-specific. With regard to **chapters 3** and **4**, we investigated brain homogenates of the regions of interest leaving us with a mixture of neuronal and non-neuronal cells of different type and function. As a consequence, observed effects might be merely due to an altered cell-type composition, a question addressed in **chapter 6**, or differences might not be detected due to counteracting cell-types, or simply due to noise, concomitant with heterogeneous tissue samples. Moreover, cell-type specificity might be important with regard to the neural activation in **chapter 5**, investigating, for example, the amygdala subnuclei, which have been shown to comprise a number of distinct subpopulations that serve distinct functions (Asan, Steinke and Lesch, 2013). Similar to the overlapping or counteracting effects, mentioned for RNA expression and epigenetic marks, cell-type specific activation patterns can be washed out by unspecific investigation or, alternatively, introduced by alterations in cell-type composition.

Future research investigating, the role of 5-HT signalling, its acute involvement in stress reactivity, and its role in developmental priming, should therefore aim to, firstly, identify the affected brain circuitry and cell populations and, secondly, in a targeted approach, identify functionality and susceptibility of those cells towards genetic and/or environmental variations. To gain a broader understanding of the epigenetic mechanisms involved, at least the most common epigenetic marks should be investigated in order to help generating an integrative, holistic view. However, more important is the validation of possible causal associations and, to that end, technologies such as epigenetic editing and optogenetics should be undertaken, once eligible targets have been identified.

In conclusion, we were able to link 5-HT deficiency to specific traits and to differential susceptibility to early adversity. Molecular observations, regarding, on the one hand, gene expression as well as epigenetic programming and, on the other hand, functional morphology, are suggesting the interplay of acute 5-HT system dysfunction and, developmentally induced or inherited, chronic dysregulation of the 5-HT- and stress-system, in concerting the phenotypic outcome.

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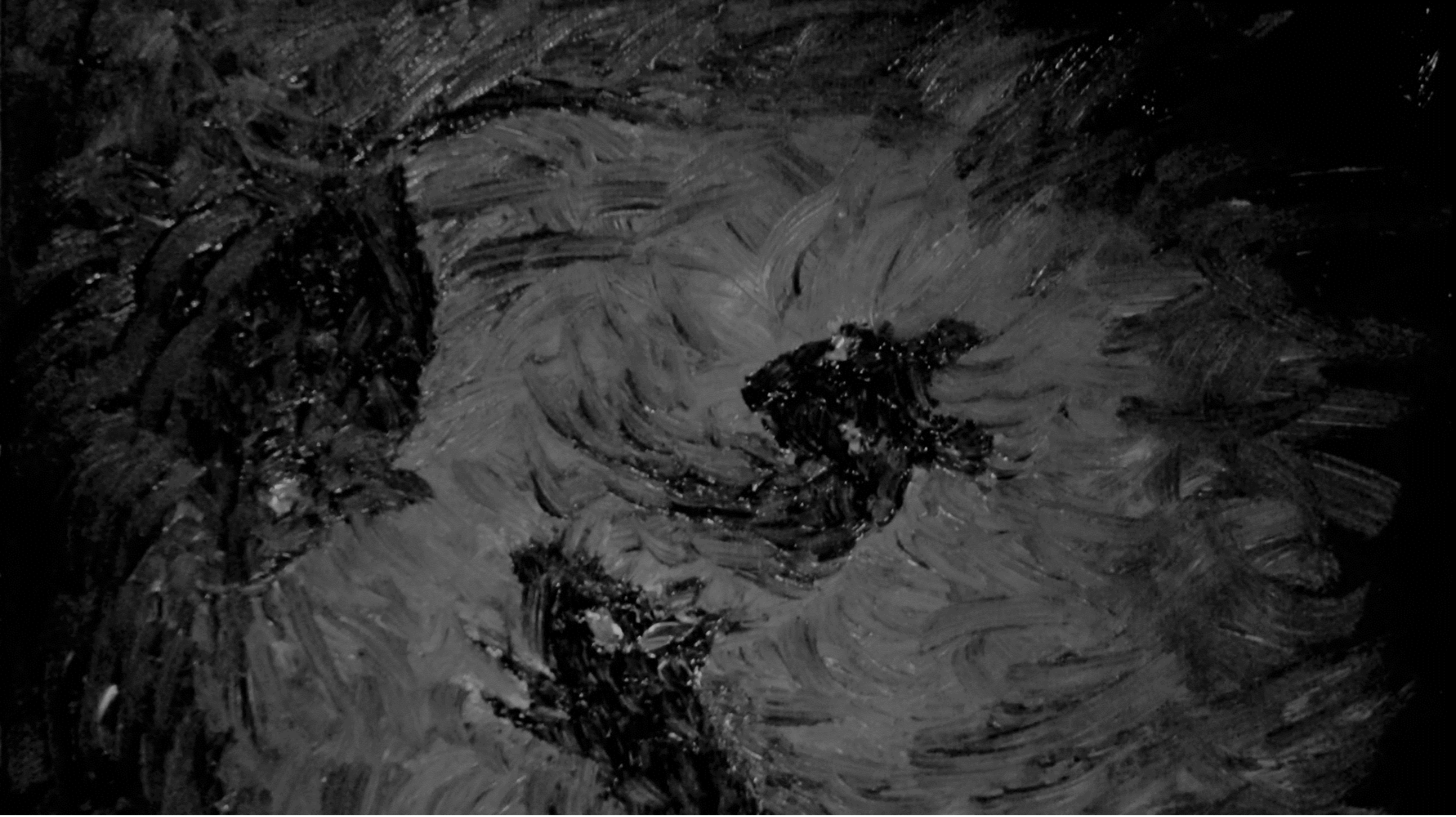
Chapter VII
General discussion



Appendix

Summary

The work presented in this thesis covers the effects of early-life adversity in the context of altered serotonin (5-HT; 5-hydroxytryptamine) system functioning in mice. The main body is focussing on a screening approach identifying molecular processes, potentially involved in distinct behavioural manifestations that emerge from or are concomitant with early adversity and, with regard to some behavioural manifestations, dependent on the functioning of the 5-HT system.

Chapter 1 gives a short conceptual introduction into gene-by-environment interactions, with a focus on early adversity and genetic variants of the 5-HT system. Furthermore, it introduces epigenetic modifications at the DNA strand itself as well as at histone tails as potential link between genetic variation and experiences throughout life. This introduction is followed by a brief outline of this thesis.

In **chapter 2**, we give an overview on the physiological and molecular footing of aggressive behaviour. Moreover, the chapter discusses the aetiology of violent manifestations and points out the vast heterogeneity of identified correlates, making the search for a unitary cause pointless. One line of work, found that molecular changes, induced and affected by early-life adversity, promote aggression-related pathology and that many of the observed changes were related to changes in the 5-HT system. The 5-HT system is suggested to serve as a distinctive regulator of aggressive behaviour related to exaggerated, sociopathy-like aggression. Furthermore, we point out that the stress-induced alterations in 5-HT system functioning are most likely brought about by and also causally involved in inducing epigenetic modifications. Finally, the importance of more integrative working models is emphasised, suggesting future work to focus more on unravelling gene accessibility and consequent transcriptional activity by examining chromatin as regulatory machinery that integrates multiple layers of environmental input over time.

Chapters 3 and **4** report findings of behavioural and molecular screening studies, following gene-by-environment interactions involving diverse components of the 5-HT system in the context of adversity during relevant developmental periods. In **chapter 3**, consequences of the reciprocal relationship of 5-HT and early adversity were investigated using a tryptophan hydroxylase 2 (*Tph2*)-deficient mouse line (Tph2 x MS). *Tph2* null mutant ($Tph2^{-/-}$) and heterozygous ($Tph2^{+/-}$) mice, resulting in full or partial depletion of brain 5-HT, respectively, as well as their wildtype littermates were exposed to neonatal maternal separation (MS). Subsequently, adult, male offspring were screened for behavioural, physiological and molecular changes. The focus of the behavioural screening was on behaviours of emotional dysregulation, such as anxiety and aggression. In this study we found that the full 5-HT depletion in $Tph2^{-/-}$ mice profoundly affected most of the investigated behaviours, body-weight and the presence of faecal corticosterone metabolites under home cage conditions. $Tph2^{-/-}$ mice showed increased locomotor activity, decreased anxiety in an aversive context, decreased social behaviour and a more pronounced stress response to behavioural testing. Wildtype offspring, showed a behavioural profile profoundly distinct from that observed in TPH2-deficient mice. This difference was

found to be washed out following MS, in particular, with regard to aggression-related behaviour and activity. Interestingly, *Tph2*$^{+/-}$ offspring showed an ambiguous profile, either intermediate or comparable to wildtype or *Tph2*$^{-/-}$ mice, dependent on the investigated task. On the molecular level *Tph2* genotype and its interaction with MS were associated with distinct changes in RNA expression. A subset of differentially expressed genes (3%) showed an overlap with the specific DNA methylation profiles at corresponding loci. A promising candidate arising from this analysis was the hormone cholecystokinin, which can also act as neuropeptide and was furthermore associated with the observed anxiety behaviour in the current study.

In **chapter 4**, we were focussing on differential susceptibility to early adversity. This was investigated in the context of a 5-HT transporter (*5-Htt*) genetic variant. Resilience is the key towards mental well-being and a wealth of research suggested that 5-HT system-dependent mechanisms might be involved. In humans, the *5-HTT* gene-linked polymorphic region (*5-HTTLPR*), in particular, proved to be of relevance in modifying the outcome of early adversity. Therefore, we chose to use offspring of a *5-Htt*-deficient mouse line, in order to investigate the effects of prenatal stress, with a focus on differential susceptibility and associated molecular changes. A behavioural screening in these mice, with focus on anxiety-related and depressive-like behaviours, revealed that, dependent on the *5-Htt* genotype, offspring of PS dams showed distinct reactivity to the elevated plus maze and the 3-chamber sociability test. Furthermore, differential susceptibility to PS was associated with differential gene expression in the hippocampus. Similar to the behavioural observations, the distinct expression profile was found to be dependent on the *5-Htt* genotype, with up to 30 times more genes regulated in *5-Htt*$^{+/-}$ offspring when compared to *5-Htt* $^{+/+}$ offspring. These observed molecular changes were in particular related to genes, involved in oligodendrocyte differentiation and myelination. Besides the gene expression profile, we determined genome-wide H3K4me3 profiles. As for the RNA expression, the observed changes were distinctive for genotype and susceptibility, but showed only marginal overlap with the determined, differentially expressed genes.

In **chapters 5** and **6**, a more targeted approach was employed. To further investigate the morpho-functional consequences of early adversity, we employed an c-Fos immunohistological staining in **chapter 5**. This study was aimed at further elucidating the suggested interaction of 5-HT and MS with regard to the effects on anxiety-related behaviours. As described for **chapter 3**, we used *Tph2*$^{-/-}$, *Tph2*$^{+/-}$ and wildtype offspring, exposed or not exposed to neonatal maternal separation and performed anxiety-related tests followed by neural activity imaging during adulthood. Interestingly, MS effects were only found in *Tph2*-deficient animals. In both anxiety tests MS promoted a more active approach in brain 5-HT-deficient offspring. On the neural level, we observed an activation in most of the investigated regions of the fear circuitries. However, some of these regions also showed a distinctive activation pattern, dependent on the test, *Tph2* genotype and/or MS, suggesting the involvement of specific circuitries and susceptibility to the different tasks and predispositions.

In **chapter 6**, we aimed to validate a candidate gene, i.e. myelin basic protein (*Mbp*), which we previously linked to PS. The effects of PS on the expression and methylation of *Mbp* were furthermore found to be dependent on the genetic variation of *5-Htt*. In the current study, we investigated the DNA methylation profile in both, human infants and adult mice, in the context of maternal experiences and

emotions during pregnancy. In mice, we furthermore determined the expression of two distinct forms of *Mbp*. In humans, early adversity was examined by use of stress- and anxiety-related questionnaires and cortisol measures. Methylation at the *MBP* locus was investigated in umbilical cord blood of the offspring. Pregnant mice were exposed to restraint stress and offspring, either heterozygous for a *5-Htt* gene variant, or wildtype, were analysed with regard to *Mbp* mRNA expression, *Mbp* methylation and myelin-related, histological parameters in the hippocampus. Interestingly, the investigation of *MBP* methylation in both species seemed to be susceptible to early programming, irrespective of the tissue investigated. In human infants, an increase of the maternal cortisol was positively related to *MBP* gene methylation at one of two investigated CpG sites. In mice, PS-induced, reduced weight gain during the stress period was associated with an increased methylation at a previously identified CpG site at the *Mbp* gene in wildtype but not in *5-Htt*-deficient offspring. As for the histological approaches, only a genotype dependent effect was observed, suggesting that the observed altered level in DNA methylation might represent DNA priming and only take effect in the context of further challenge.

Finally, in **chapter 7** the experimental results, presented in this thesis are critically discussed and put into perspective. Limitations of the conducted studies and potential future directions are highlighted. Overall the results of our studies emphasize the critical role 5-HT plays in the modulation of early adversity. The results await further research dissecting the identified candidate genes and pathways.

Zusammenfassung

Diese Arbeit berichtet Ergebnisse der umfassenden Erforschung des Einflusses von aversiven Bedingungen während der pränatalen und frühkindlichen Entwicklungsphase, unter dem Einfluss genetischer Variationen des serotonergen (5-HT, 5-Hydroxytryptamin) Systems, im Mausmodel. Der Hauptfokus der Thesis lag bei der hypothesenfreien Untersuchung der Konsequenzen, die durch den kombinierten Effekt aversiver Bedingungen, und genetischer Prädisposition ausgelöst werden, sowie, final, der Ermittlung potentieller Kandidatengene, die an der Manifestierung verhaltensbezogener Konsequenzen beteiligt sein können sowie durch epigenetische Mechanismen reguliert werden.

Kapitel 1 führt kurz in den konzeptionellen Entwurf von Gene-Umwelt-Interaktionen ein, mit einem Fokus auf die pränatale und frühkindliche Periode und 5-HT relevante genetische Prädispositionen. Des Weiteren werden epigenetische Modifizierungen am DNS-strang selbst oder an den Enden der Histonstränge als mögliche Brücke zwischen Umwelt und Genom vorgestellt. Kapitel 1 wird abgeschlossen durch eine Zusammenfassung der vorliegenden Arbeit.

In **Kapitel 2** wird zunächst die physiologische und molekulare Basis aggressiven Verhaltens zusammengefasst, gefolgt von einer Diskussion möglicher Auslöser übersteigerter Aggression. Zudem wird das komplexe Wesen identifizierter Auslöser dargestellt. Die Mannigfaltigkeit potentiell kausaler Kandidatengene und Mechanismen ist überwältigend und verhindert die Determinierung einzelner, kausaler Komponenten. Eine potentielle Forschungsrichtung, die sich mit abnormer Aggressivität befasst, erforscht die molekularen Veränderungen, die durch das Erfahren pränataler oder frühkindlicher, negativer Erlebnisse ausgelöst werden. Diese Veränderungen scheinen Aggressivität zu fördern und sind, unter Anderem, mit Veränderungen im 5-HT-system assoziiert. Auch, wurde das 5-HT-system als einer der regulatorischen Faktoren identifiziert, die übersteigerte Aggression und den zweckorientierten Gebrauch von Aggression, wie er bei Psychopathie beobachtet wird, spezifisch unterscheidet. Des Weiteren wird in dieser Review diskutiert, dass die beobachteten Änderungen im 5-HT-system sowohl kausal für, als auch Konsequenz von epigenetischen Modifizierungen sind. Extensive Forschung an Gen-Umwelt-Interaktions Modellen unterstreicht die Vielschichtigkeit epigenetischer Regulation, die sich in den komplexen Gen-Umwelt- und Gen-Gen-Umwelt-Interaktionen, sowie den kumulativen Effekten verschiedener Umweltaspekte wiederspiegelt. Zukünftige Forschung muss daher einen ganzheitlichen Ansatz anstreben, der Chromatin als regulatorische Interaktionsfläche begreift, die Erfahrungen in Regulation umwandelt und so die epigenetische Landschaft eines Individuums definiert.

In **Kapitel 3** und **4** werden die Ergebnisse zweier hypothesenfreier Untersuchungen beschrieben, die sich jeweils auf einen anderen Aspekt des 5-HT-systems, sowie andere Entwicklungszeitpunkte bezogen. In **Kapitel 3** wurde der Zusammenhang frühkindlicher Deprivation mit dem physiologischen Mangel an 5-HT im Gehirn erforscht. Zu diesem Zweck wurden Mäuse, die eine genetische Ablation des Gens für Tryptophan-Hydroxylase 2 (*Tph2*) haben, und somit kein 5-HT im Gehirn synthetisieren können, einem Paradigma frühkindlichen Entzugs der mütterlichen Fürsorge ausgesetzt. Homozygot defiziente Welpen (*Tph2*$^{-/-}$), heterozygot defiziente Welpen (*Tph2*+/-), sowie deren wildtypische Geschwister, wurden während der ersten zwei Lebenswochen für mehrere Stunden am Tag von der Mutter getrennt. Im Erwachsenenalter wurden diese Tiere dann auf Verhaltensauffälligkeiten und

physiologische sowie molekulare Effekte untersucht. Die untersuchten Verhalten waren vor Allem auf Verhalten emotionaler Dysfunktion, wie zum Beispiel Angstverhalten und Aggression, begrenzt. In dieser Studie beobachteten wir, dass ein vollständiger Mangel an 5-HT im Gehirn schwerwiegende Konsequenzen auf die beobachteten Verhalten, das Körpergewicht der Tiere, sowie deren Corticosteron-metabolit Spiegel in Faeces hat. Die beobachteten Effekte waren unabhängig von der frühkindlichen Stresserfahrung. *Tph2*$^{-/-}$ Tiere waren aktiver, weniger angstinhibiert und weniger sozial als ihre wildtypischen Geschwister. Auch, zeigten die vollständig 5-HT defizienten Tiere einen deutlich erhöhten Corticosteron-metabolit Spiegel unter basalen Bedingungen. Das beobachtete Verhalten in wildtypischen Geschwistern schien sich jedoch nach der frühkindlichen Separation von der Mutter an das, in 5-HT defizienten Tieren beobachtete, Verhaltensprofil anzugleichen. Heterozygote Nachkommen zeigten ein sehr zwiespältiges Profil, welches einmal dem Profil in *Tph2*$^{-/-}$ Tieren und einmal dem Verhalten, welches in wildtypischen Tieren beobachtet wurde, glich. Zudem wurden auch intermediäre Level in *Tph2*$^{+/-}$ Tieren beobachtet. Auf der molekularen Ebene hatten, sowohl Tph2 Defizienz, als auch die Interaktion zwischen Tph2 Defizienz und frühkindlichem Stress, spezifische Auswirkungen. Ein Anteil an Genen, deren Expression durch den Genotyp, oder dessen Interaktion mit Stress beeinflusst war, war auch mit einem differentiell methylierten *Locus* assoziiert. Etwa 3% aller diferentiell exprimierten Gene zeigten eine solche Überlappung und besonders eines dieser Gene schien vielversprechend als potentieller Effektor frühkindlicher Deprivation und 5-HT Defizienz, da es nicht nur durch die gleichen Faktoren in Expression und Methylierung beeinflusst wurde, sondern zudem, sowohl Genexpression, DNS Methylierung als auch Angstverhalten miteinander korreliert waren. Das *Cck* Gen kodiert das Neuropeptid Cholecystokinin. Cholecystokinin ist ein Hormon des Gastro-Intestinal-Trakts und erfüllt auch eine Rolle als Neuropeptid im Gehirn, wo es mit Angstverhalten assoziiert ist.

In **Kapitel 4** konzentrierten wir uns auf differentielle Suszeptibilität zu pränatalem Stress im Kontext einer genetischen Variante des 5-HT Transporters (5-Htt, Sert, Slc6a4). Verringerte Suszeptibilität, die auch als Resilienz bezeichnet wird, ist der Schlüssel zu psychischer Gesundheit. Viele Studien, die sich die Erforschung von Resilienz zum Ziel gesetzt hatten, fanden Grund zu der Annahme, dass das 5-HT-system dabei eine Rolle spiele. Der 5-HTT Längenpolymorphismus (5-HTTLPR) wurde dabei zu einem Subjekt besonderen Interesses in Studien, die solche Suszeptibilität in Menschen untersuchten. In unserer Studie verwendeten wir daher Tiere einer *5-Htt* defizienten Mauslinie und untersuchten die Nachkommen wildtypischer Mütter, die von einem heterozygoten Männchen geschwängert worden und vom Tag 13 bis zum Tag 17 der Schwangerschaft einem Stressparadigma ausgesetzt worden waren, im erwachsenen Alter. Die erwachsenen Nachkommen wurden in Bezug auf Verhalten, sowie physiologische und molekulare Effekte untersucht. Die Verhaltenstests zeigten, dass abhängig vom *5-Htt* Genotypen, unterschiedliche Verhaltensprofile beobachtet werden konnten. Am auffälligsten war die hohe Varianz in pränatal gestressten Tieren in zwei Angsttests, der eine mit Fokus auf aversive Stimuli, der andere mit Blick auf soziale Interaktion. Das aufteilen der Tiere in Suszeptibilitätsgruppen zeigte im Folgenden, dass in wildtypischen Nachkommen pränataler Stress zur Verringerung sozialen Verhaltens führte, dass jedoch in den selben Tieren, in denen eine Verringerung des Sozialverhaltens beobachtet wurde, auch eine Verringerung des Angstverhaltens in einer aversiven Umgebung beobachtet werden konnte. In *5-Htt* defizienten Tieren war dieses Verhältnis umgekehrt und die sozial normalen, gestressten Nachkommen zeigten auch ein verringertes Angstverhalten in der aversiven

Umgebung. Diese differentielle Suszeptibilität war zudem mit einem spezifischen Genexpressionsprofil im Hippocampus assoziiert. In *5-Htt* defizienten Nachkommen waren 30-mal mehr Gene reguliert in ihrer Expression als in ihren wildtypischen Geschwistern und auch waren viele der geänderten Gene assoziiert mit der Differenzierung von Oligodendrozyten, sowie der Myelinisierung *per se*. Die zusätzlich durchgeführte Analyse der immunbasierten Anreicherung der Histon-modifikation H3K4me3 zeigte zwar spezifische Profile für die genotyp-spezifische, differenzielle Suszeptibilität, aber nur geringe Überlappungen mit dem Genexpressionsprofil.

In **Kapitel 5** und **6** wendeten wir zielgerichtetere Methoden an um die morpho-funktionellen Konsequenzen pränatalen und frühkindlichen Stresses genauer zu erforschen. Zu diesem Zweck benutzen wir immunhistologische Techniken. In **Kapitel 5** wurde die neurale Aktivität mit Hilfe eines anti-c-Fos Antikörpers untersucht. In dieser Studie wollten wir die Interaktion zwischen 5-HT und frühkindlichen aversiven Erfahrungen weiter erforschen, ins besondere im Hinblick auf Angstverhalten. Wie in der vorherigen Studie, beschrieben in **Kapitel 3**, benutzten wir in dieser Studie *Tph2*$^{-/-}$ und *Tph2*$^{+/-}$ Tiere, sowie wildtypische Wurfgeschwister, die frühkindlicher Deprivation ausgesetzt wurden. Diese Tiere wurden im Erwachsenenalter schließlich einem, von zwei spezifischen, Angsttest unterzogen und die neurale Aktivität, die durch ebenjene Angsttests ausgelöst wurde, wurde untersucht. Effekte maternaler Deprivation wurden nur in Tph2 defizienten Nachkommen beobachtet. In beiden Angsttests förderte maternale Deprivation eine aktivere Stressbewältigung. Auch hatten beide Angsttests die Aktivierung neuraler Zellen in allen untersuchten Gehirnregionen des Angstsystems zur Folge. In manchen Regionen war diese Aktivierung jedoch spezifisch für entweder den Tph2 Genotypen oder MS, abhängig vom verwendeten Angsttest. Diese Beobachtung legt eine spezifische Rolle sowie Suszeptibilität bestimmter Gehirnstrukturen nahe.

In **Kapitel 6** war das Ziel die Validierung eines vormals identifizierten Kandidatengens, welches im Zusammenhang einer Gene-Umwelt-Interaktion des 5-HTT mit pränatalem Stress verändert gefunden worden war, nämlich „Myelin basisches Protein“ (*MBP*). In dieser Studie untersuchten wir das DNS-Metylierungsprofil in humanen Kindern sowie in adulten Mäusen im Zusammenhang mit pränatalem Stress. In den Mäusen untersuchten wir zudem die RNA-expression zweier Isoformen des *Mbp* Gens. Die Natur und der Schweregrad des pränatalen Stresses wurden in den Menschen durch spezifische angst- und depressionsbezogene Fragebögen abgefragt. Zusätzlich wurde das Cortisol im Speichel gemessen. Des Weiteren wurde die DNS-Methylierung an einem *Locus* des *MBP* Gens an DNS aus Nabelschnurblut untersucht. In der Maus-Studie, wurden Nachkommen wildtypischer Mütter, die von einem heterozygoten Männchen geschwängert worden und vom Tag 13 bis zum Tag 17 der Schwangerschaft einem Stressparadigma ausgesetzt worden waren, untersucht. Das DNS-Methylierungsprofil, sowie Expression zweier Isoformen und Protein wurden im Hippocampus der adulten Tiere erforscht. Unabhängig von der untersuchten Spezies, konnte eine Suszeptibilität, der DNA methylierung zur pränatalen Umgebung festgestellt werden. Im Menschen war ein erhöhter Cortisol-spiegel während der Schwangerschaft mit erhöhter *MBP* Methylierung an einem der zwei untersuchten CpGs assoziiert. Dies war nur in weiblichen Nachkommen beobachtbar. In der Maus-Studie, war die verringerte Gewichtszunahme, die in gestressten Müttern beobachtet wurde, mit einer erhöhten Methylierung an dem vormals identifizierten Locus in wildtypischen, nicht jedoch in *5-Htt* defizienten Nachkommen, assoziiert.

Kapitel 7 stellt die Diskussion aller, in der Thesis präsentierten, Daten dar. Die Ergebnisse werden kritisch diskutiert und ins Verhältnis gesetzt mit der Arbeit anderer Forscher. Des Weiteren werden limitierende Faktoren, sowie mögliche zukünftige Forschungsrichtungen aufgezeigt. Alles in allem unterstreicht die Arbeit, die in dieser Thesis dargestellt wird, die zentrale Rolle des 5-HT-systems in der Modifikation von frühen Lebenserfahrungen. Auch bilden die Ergebnisse dieser Arbeit die Basis für weitere, zielgerichtetere Forschung, mit einem Fokus auf die in dieser Studie identifizierten Gene und Mechanismen.

Valorisation

Psychopathological manifestations, such as fear and anxiety disorders in the form of posttraumatic stress disorder (PTSD) and generalised anxiety disorder (GAD), or aggression and impulsivity are a major social burden in today's society (Penden, McGee and Krug, 2000; World Health Organization, 2001, 2002; Kessler and Greenberg, 2012; American Psychiatric Association, 2013; Chisholm *et al.*, 2016). Symptoms, including excessive worry, avoidant behaviour or panic attacks as well as inflicting harm on others or self, impair patients in everyday life and are putting a strain on communities and families. Next to the social component, the economic burden, imposed by the treatment of symptoms and dealing with direct and indirect consequences of the disorders, is substantial. According to the global burden of disease estimates, undertaken in the 1990ies, mental disorders are amongst the most costly disorders, causing up to 10.5% of all disability-adjusted life years (DALYs) lost (Murray and Lopez, 1996). A foremost reason for this unproportional representation of mental disorders in the global burden of disease estimates, might be found in the historic stigma, accompanying mental disorder and concomitant behavioural aberrations. In consequence, insufficient means and treatment have been provided giving way to secondary consequences, such as long-term disability, resulting in unemployment, or anxiety-related underemployment (World Health Organization, 2001; Kessler and Greenberg, 2012) and, in the case of pathological aggression, in injury and e.g. sexually transmitted diseases (World Health Organization, 2002). For both patients and society, it is relevant to extend the current understanding of the underlying brain circuitries and molecular mechanisms of overshooting fear and anxiety, antisocial behaviour and other related traits, in order to promote more efficient, targeted, and individual treatment options.

Only in recent years, mental disorders have been accepted as vital contributor to an individual's health and well-being. Simultaneously, the field of neuroscience has experienced a boost, making use of advances in imaging techniques and in the field of genetics and molecular biology. In particular, one line of research proofed promising in closing in on the roots of mental disorders, by focussing on a joint approach of gene-environment-based psychiatric research and neuroscience (Caspi and Moffitt, 2006). A prominent factor that has emerged from all related investigations and was identified as one of the first proofs of this combined approach is the serotonin (5-hydroxytryptamine, 5-HT) system. The 5-HT system comprises multiple levels of effect and exerts a multitude of functions throughout development (Gaspar, Cases and Maroteaux, 2003). It is known as neuromodulator and regulates the excitability of neurons (Andrade, 1998). Furthermore, genetic variants that were identified in genes, encoding relevant factors of the 5-HT system were shown to be heritable factors in a multitude of psychopathologies (Gaspar, Cases and Maroteaux, 2003). 5-HT has been associated with pathological traits across diagnosed mental disorders, including depression and anxiety disorder, aggression and impulsivity (Apter *et al.*, 1990). Next to the heritable alterations in 5-HT function, early adversity had been shown to take effect on 5-HT system regulation (Booij, Szyf, *et al.*, 2015). In this respect, the approaches used in the current thesis were aimed to answer, at least in part, some of the most relevant questions, regarding the involvement of the 5-HT system in the aetiology and manifestation of such disorders in rodent models of genetic variation. Animal models represent a valuable tool towards the identification of potential candidate genes and affected pathways, mechanisms and circuitries.

Taken together, research reported in the previous chapters supports a role of 5-HT in modulating the effects of early adversity through epigenetic programming of brain developmental trajectories and, consequently, behaviour. A large body of work has already been focused on specific pathways of developmental programming (Booij, Richard, *et al.*, 2015) and acute 5-HT signalling (Paul and Lowry, 2013; Paul *et al.*, 2014). However, only few studies were focussing on the effects and mechanisms in animals lacking brain 5-HT, which allows the determination of, if and how 5-HT is involved in the observed effects of early adversity, which is making our model uniquely fitted to investigate behavioural disorders that emerge in the context of early adversity. The genetically modified mouse models, used in this thesis allow investigating molecular and neuronal correlates associated with altered brain 5-HT system functioning and allows to compare the effects of MS in wildtype animals and mice with an altered 5-HT system. Furthermore, in light of the previously described importance of 5-HT signalling throughout development and its involvement in manifold disorders of the mind, the current study provides a valuable tool to identify potential targets for interventional studies. Identifying molecular markers, neural activation and, consequently, affected subpopulations in specific brain regions. The subregions of the brain each fulfil tremendously complex functions that are subject to reciprocal regulation (Bullmore and Sporns, 2009). Each region consists of subregions, which consist of clusters of specific neurons that can be distinguished by co-expression of certain markers, morphology and electrophysiological characteristics (Hale and Lowry, 2011; Asan, Steinke and Lesch, 2013), which stresses the highly distinctive nature of neuronal signalling. Moreover, despite increasing knowledge in all relevant fields of neuroscience, there still is a deep lack in interdisciplinary knowledge utilisation, which deprives the research community of a wholistic view. Nevertheless, the complexity and comprehensive nature of the involvement of 5-HT in all aspects of an individual's health, brain circuitry and even gene expression regulation becomes more and more apparent, underscoring an ambiguous role of 5-HT, dependent on time and region of exposure/regulation (Gaspar, Cases and Maroteaux, 2003). Current treatments, however, usually target factors unspecifically over the whole organism. One impressive example of such untargeted approaches is the class of selective serotonin reuptake inhibitors (SSRIs). Components of the family of SSRIs target the 5-HT transporter and, in the brain alone, cause a multitude of effects through prolonged dwelling time within the synaptic cleft and, consequently, increased exposure of 5-HT receptors to their substrate (Ferguson, 2001). Amongst the observed side effects are: sexual dysfunction, weight gain, and sleep problems. Furthermore, more often than not, those treatments are ignorant of effects of genetic variation on the treatment. For example, SSRI side effects were associated with genetic polymorphisms in the 5-HT transporter and receptors (Garfield *et al.*, 2014) as well as for example effects of genetic variations on metabolism of the administered drug (Probst-Schendzielorz, Viviani and Stingl, 2015). Side effects like this are making a more targeted approach preferable to unspecific blockage or activation. In addition, serotonin signalling might represent the consequence and not the cause of neurological alterations, leading to mental disorder (Nordquist and Oreland, 2010), making research on developmental processes, and how genetic alterations and early adversity interfere with such trajectories, vital for the successful determination of acute targets.

To unravel the involved mechanisms in specific brain regions and the physiological consequences of such alterations would finally allow a purposeful intervention, minimising side effects. The work in this thesis addressed the question of gene-by-environment interaction effects on the activation of fear

circuitry as well as expression and expression regulation on a genome-wide level. In the course of this work we identified the amygdala as important hub of early adversity, which interacts with hind brain regions in a 5-HT-dependent manner, regulating behaviour. Moreover, gene expression in limbic brain regions was found to be regulated by early adversity, dependent on 5-HT, and several specific candidate genes were discovered in genome-wide approaches. Thus, the work conducted in this thesis represents a hypothesis-generating approach, pointing out several lines along which future research can develop. Target-specific manipulations in animals, using techniques, such as optogenetic gene activation or inhibition, or stimulation of specific subpopulations of neurons, or genetic as well as epigenetic editing will represent some of the next steps on the way towards more sophisticated approaches to treat mental disorder. Taken together these findings may lead to interventional strategies on the molecular level and to help guiding more invasive therapies, such as deep brain stimulation fitted for the individual patient based on their molecular make-up.

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Daniël, you have been the rock amongst my supervisory team. You accompanied me throughout the worst and the best of times during the last 4 years. I could always rely on finding an open ear for my problems and a helping hand for any trouble and even though you were always there, you never micromanaged me and my projects. I deeply appreciate your lively character and the way you always point out the positive and the way forward. Given that I am a very reserved person, more critical and negative than anything, I might have gotten stuck several times, if it wasn't for your positive reinforcement and encouragement. You tend to say that science is your fourth child and as your student I was able to experience the devotion you have for the research that we conduct; the enthusiasm you find for the bleakest results and the patience you have. I was lucky having you in my supervisory team.

To Professor Harry Steinbusch, I want to thank you for the opportunities you put in my way. Thanks to you, I was able to spent 6 months at Oxford university, which I can describe as a critically important time on my way to becoming an independent and critical thinker. Although you were not directly involved in my projects, your input was always valuable.

To Professor Sauer, as part of my Würzburg promotion committee, I want to thank you for the patience with my special situation, as the legal parties did not make it easy on us to gain a joint degree.

Daniel and Suzie, I want to thank you for an amazing time in Oxford. Daniel, the time in your lab taught me a lot; your critical and fast way of thinking challenged me in a very positive way and I would think contributed significantly to the way I am now as a scientist. My time in Oxford was the most creative time during my PhD, in terms of scientific planning and thinking and the way science is conducted there represents for me the original spirit of science.

Angelika, I have known you for a very long time now, and after doing my Bachelor thesis in your group, neuroscience got me hooked. Since then, I have always seen you as the role model of an exemplary and upright scientist. Your unfaltering honesty and clarity serve me as an example and even though we have not officially been linked as such, I see you as a mentor.

Furthermore, I want to thank all the PIs that were involved in the generation or analysis of my data: To Gunter for his patient support with my EIA, to Professor Rupert Palme, many thanks for the introduction into, and the support with the determination of the faecal metabolic corticosterone levels, to Professor Tatyana Strekalova, I want to thank you for the hands-on introduction into animal research, to Konrad, thank you for the unfaltering support with my sequencing data.

Secondly, next to all the PIs involved, I would like to specifically and warmly thank the technician team in Würzburg:

Liebe Gabi, selten bin ich einer Person begegnet, die eine solche grandiose Ausstrahlung, Motivation und Genauigkeit besitzt wie du. Mit dir zu arbeiten zählt zu meinen besten Laborerfahrungen. Die Gründlichkeit, mit der du eine jede Technik hinterfragst und erarbeitest war mir ein großes Vorbild und man kann sagen, dass du mich im Labor geformt hast. Nach dir habe ich nie wieder so effizient mit einer Person gearbeitet, es war fast als wären wir eine Person mit vier anstatt zwei Händen und ich bin aus vollstem Herzen dankbar, dass ich von dir lernen und mit dir arbeiten durfte. Auch möchte ich mich für viele aufbauende und interessante Gespräche über einer Tasse Kaffee oder Tee bedanken und hoffe, dass es uns bald mal wieder gelingt uns irgendwo zusammen zu setzen um uns alle Neuigkeiten zu erzählen. Ich drück dich feste, vielen Dank für alles!

Liebe Inge, auch dir möchte ich für eine ganz besondere Zeit danken. Du hast mich unter deine Fittiche genommen, nachdem ich nach einem Jahr plötzlich aus der Histo ins PCR-Labor kam. Es waren ja schon viele Verdächtige aus der Histo rüber gekommen, aber du hast mir mit aller Geduld und viel Wissen die hohe Kunst des Genotypisierens beigebracht. Neben meinen Uni Kursen war dies das erste Mal, dass ich vollen „Körperkontakt“ mit Genetik hatte, eine prägende Erfahrung… Die Jahre, in denen ich mit dir als HiWi gearbeitet habe waren toll und auch bin ich dankbar, dass ich nicht nur auf DNA-extraktionen angesetzt wurde, sondern auch komplexere Aufgaben erfüllen durfte in dieser Zeit. Ich habe viel gelernt und zehre noch heute von diesem Wissen.

Carola, wie auch unsere Inge hast du maßgeblich zu meiner Bildung in technik-relevanten Dingen beigetragen. Zudem möchte ich mich bei dir für eine grandiose Zeit bedanken, in der wir auch neben der Arbeit einige Abenteuer bestritten hatten.

Alle anderen TAs, Thomas, Schnicki, Marion, Nicole und Katharina: danke für eure Unterstützung! Ohne euch hätte ich es nicht geschafft.

Next to the tech team in Würzburg, my thanks also go to the tech team in Maastricht, in particular Hellen, who took it upon her to support me in many of my endeavours and Barbie, who was always there in case any support was needed and who worked her way through the mysterious faecal

metabolic corticosterone EIA for me and of course all the others, that were providing support in the background. Heel erg bedankt!!!

Judith, ever since I started at the department, you were a great support, facilitated a lot of things and made the impossible possible! I would probably not be here today without you and your work was invaluable in particular with the funny position of a shared PhD student. This can very often cause trouble but thanks to you almost nothing went ever wrong.

Rachelle, as the Dutch counterpart to Judith, we only met later and I think you had less trouble with my shared position, but nevertheless your constant support during my time in Maastricht, ensuring I wouldn't get kicked out of my mail account, 24 hour access after I dared to give my UM card to Johanna and on many more occasions was a great help.

Next, I would like to thank all my colleagues and friends. To make it easier on my mind and to avoid forgetting anyone (which might still happen, in that case please forgive me), I will thank you all in chronological order.

Dear Karla, you already accompanied me through my master studies, as daily supervisor. Your quick mind and critical approach, not only in the lab but overall were very impressive. Over time we also became friends and I learned to appreciate your great cooking skills and became very impressed with your handiwork. It was a sad day, when you left our group and a cutting loss regarding knowledge. I always hoped I was able to catch up on the lost knowledge, but who knows where we would be today if you could have stayed. Many thanks for being a great tutor and example.

Julia, like Karla, we already met during my Masters and immediately hit it off, we were both very fond of nice dresses and shoes, so an immediate link. Next to your scientific brilliance (I rarely ever met someone to perform the tasks you do, as effortlessly as you do) you were great company.

Sarah, I also want to thank you. I always appreciated your honesty. You were always truly hard working and served me as great example.

Nicole, you are a true scientist. You worked the hardest and you are successful. I remember, how very often the two of us would be alone in the office, I curating my "kill list" for the next day and you submitting an article or reformatting your review, and all of this, next to learning a new technique, and supervising Ruslan. Also, you have the most beautiful and accurate lab journals I have ever seen! Next to being this awesome scientist, you are also a great person, funny and spontaneous. Thank you for a great time in Würzburg and for great Whatsapp conversations ever since. I am grateful to call you a friend and my Paranymph.

Christiane, you were always one year ahead of me (I think by now it is more^^) and ever since you joined the lab I appreciated your company! You were always awesome fun, I loved getting the Muck burger's together and later on also the Wohnzimmer Cocktail moments. Next to this, you are a great scientist, and on some occasions, we were already able to work together. You are always helpful and kind and I want to thank you for a great time together, being the epigenetic kids before it became popular!

Charline, I also want to thank you for being great company and an awesome person. We did not work together, but you always had an open ear, no matter how bad you were doing yourself. You always have the compassion to get angry on my behalf and to provide support wherever needed.

Inês, you were a great friend in Oxford. Thank you for your help in the lab, for your warm welcome and taking me along also after work. I enjoyed our late night dinners, after we worked until 10 or 11 PM in the lab and the fun weekends we had.

Olga, thank you for your help with my very first steps in the ZEMM, for your support in aggressotype, and an always open ear and helping hand.

Jonas, you have provided support and input and were always keen on helping hands on with whatever, from perfusions to statistics. I very much enjoy our crazy discussions about science and Tph2 mice.

Charlotte, you started off as a student but in all honesty you became a rock in the time of the animal experiments, no hour was too early, no experiment too long! You earmarked and genotyped hundreds of animals with me, went through the stress experiments and behavioural testing without complaint. We did not know weekends or holidays, we did not know sleep for when we were not in the ZEMM, we were organising the animal data, genotypes, weights, estrous and all of this under time pressure. If it wasn't for you I might have ended up upstairs, with the nice doctors. Next to that we always had an amazing time and were able to sit through between-experiment-times without dying of boredom, perfused uncountable animals and even figured out how to extract the testes. Thank you for being part of this project and for your unfaltering support.

Johanna, firstly, thank you, for being you. This said, like Charlotte, you showed bravery in the presence of an almost impossible task and nevertheless, you always remained positive. Next to your outstanding cell counting abilities, I learned to appreciate your critical mind, your joyful character and your open ways. I don't know how many times we had the most interesting, outstanding and exhilarating discussions about people, science or life itself, all of that topped with "slagroom" on a good old chocolate latte. These breaks were a tradition and some of the best time I had in Maastricht. I am very glad we took you on as PhD student because I think you are one of the people who will honour the project and my work. It was a pleasure to have you as a student and as a friend and paranymph ;).

Mark, you were the first person that talked with me when I moved to Maastricht. I always enjoyed our discussions and our lunch breaks together with Johanna. Also I want to thank you for my first (and last) Crossfit experience. My leg is still not ok :D

Roy, the very first time I met you was pretty awkward, at least I think, as I had been just there to sign my contract, but a friend of mine from Würzburg, who did her Masters with some of the former PhD students here had asked me for help with her qPCR results and dragged me through the lab. Since then I had been several times in Maastricht, before I actually moved there and I think every single time I was announced as the qPCR messiah, so I can only guess what you thought of me. But, whatever it was, I hope I was able to convince that I do not drink SYBER green for breakfast. Ever since I moved to Maastricht, I learned to appreciate your awesome taste in horror movies, video games and animes.

I always enjoyed our discussions about the Zombie-apocalypse, space scenarios and other funny topics that only ever came up when you were at lunch. Your great taste in beer and your stern personality make you very special and you have grown to be a person that I highly appreciate. Lastly and most importantly, thank you so much for your support with my sequencing data.

Artemis, I met you first during the human neuro-anatomy course, when I was just visiting Maastricht during my second year. You immediately invited me to join you and your friends and this basically represents your personality. You are a great and open person that cares so much for others. Thanks to you, I immediately felt welcome, unfortunately you left shortly upon my arrival to go to the UK, but even so your parting words were to contact you anytime I needed anything.

Marion, you are such a kind and gentle person. I greatly enjoyed our discussions in the office on science in general and publishing in particular, your support with organising the journal club and the great movie nights you organised.

Ehsan, we always had great discussions and in the end your help with my analysis on the human data was invaluable!

Lars, you had a hand full of work with all my data and I want to thank you for always trying to make time for meetings and for your constant effort to identify the best approach. You were the first computer scientist that I could nag directly and that wasn't able to run away and I learned a great deal by working with you (not R but at least I know about what is happening).

All other people of the Würzburg and Maastricht Lab that accompanied me during my PhD studies and all students that I had the pleasure of working with, thank you! You were part in making me the researcher, student, supervisor and human I am today.

Having written so much about lab members and colleagues that became friends the impression might arise that I do not have a life besides the lab, this however is not true and there were very many people that supported me throughout the not so easy phases of my PhD studies and the next section will be devoted to those people behind the curtain.

Mama, Papa, nachdem ihr mir einst nicht erlaubt hattet Philosophie zu studieren, habt ihr mich seither ohne Wenn und Aber in all meinen Karriereentscheidungen unterstützt. Wann immer ich nicht weiter wusste, wann immer Selbstzweifel an mir nagten, habt ihr mich aufgefangen und mir einen Boden unter den Füßen gegeben. Meine moralischen Werte, meine Gründlichkeit und Disziplin, sowie meine nie befriedigte Neugier wurden von euch immer gefördert, auch wenn es manchmal nicht leicht mit mir gewesen sein muss. Vielen Dank dass ihr immer da seid.

Amelie, du bist die gute Seele der Familie, wo immer du bist, schaffst du ein Heim und du willst immer das Beste für alle. Ich weiß wir haben unsere Differenzen, aber ich weiß immer, wenn ich dich brauche, bist du da.

Kili und Martin, die schweigsamen Großen, ich kann mich immer darauf verlassen, wenn jemand verhaut werden muss, dann kann ich auf euch zählen.

Annika, unsere legendären Horrorfilmabende sind mit das beste was man in Würzburg machen kann. Smarties werfen und Sneak, es gibt nichts, was den Stress besser austreibt als ein Poltergeist.

Ian, your honesty and blunt way of putting me straight has every now and then put a situation into perspective. During the last months you were probably the person that had to feel most of the moods I got by writing. Nevertheless, you always tried to make things better for me and kept me sane throughout insane stretches. Also, thank you for amazing adventure trips all over Europe and being a great friend.

Curriculum Vitae

Magdalena Theodora Weidner was born on April 16th 1989 in Würzburg, Germany, where she also grew up and obtained an education up to the university level at the St.-Ursula-Grammar School, Würzburg, which she concluded in 2008. Her majors were Biology and French (A-levels 1.8). With the University of Würzburg ranking amongst the best universities in Germany for life sciences, the choice to enroll for a Bachelor of Science program was an obvious decision, so that she enrolled for Biology in 2008. She completed her Bachelor studies within three years, submitting her Bachelor thesis, entitled "*Quantitative estimation of adult neurogenesis in mice deficient for the serotonin transporter, which went through two different learning tasks*" that was prepared in the group of PD Dr Schmitt-Böhrer, in August 2011. Following the Bachelor degree, Magdalena was able to gain a research assistant position in the laboratory of Psychiatric Neurobiology and the Bipolar Disorder Program (Work group leader Prof. Dr. Andreas Reif) at the „Klinik und Poliklinik für Psychiatrie, Psychosomatik und Psychotherapie", University of Würzburg, which ended in October 2013 with the start of her PhD studies. Next to the research assistant position, she started a two-year Biology Master program in 2011. For her research internship she joined the group of Professor Lesch, investigating the epigenetic consequences of gene-by-environment interactions in rodents. She submitted her thesis "*Effect of prenatal stress on epigenetic programming and gene expression in serotonin transporter deficient mice*" in July 2013 and concluded her degree by August 2013. During her work in this group, she also came in contact with Dr van den Hove, who, at this time point, worked in close collaboration with Professor Lesch on an early-life project. Given her close proximity to the topic and knowledge on most relevant techniques, she was offered a PhD position, to be completed in a joint effort at the University of Maastricht under the supervision of Dr van den Hove and Professor Steinbusch and at the University of Würzburg under the supervision of Professor Lesch, which she started in November 2013. During the course of her PhD studies she had several research stays abroad, from November 2013 to May 2014 she stayed at the laboratory of Dr Anthony at the department of Pharmacology at Oxford University, in June 2014, she joined Professor Strekalova in Lisbon at the Institute for hygiene and tropical medicine of the New University Lisbon to learn behavioural methods in rodents and in December 2016 she visited the laboratory of Dr Palme at the University of Veterinary Medicine Vienna to learn the determination of faecal metabolic corticosterone levels. Next to research stays Magdalena visited numerous conferences to present her work. She presented posters at the 11th meeting of the German Neuroscience Society in Göttingen in 2015, at the Dutch Neuroscience meeting in Lunteren in 2016 and at the Society for Neuroscience meeting in San Diego in 2016. Furthermore, she presented her research in a talk at the British neuroscience association meeting in Birmingham 2017 and at the NewMood revival meeting in Maastricht in 2018. During her work as a PhD student, she tutored several courses focusing on neuroanatomy and neurotransmitters at both Universities and supervised numerous Bachelor and two Master students during their internships. In the end of 2017 she won the GSLS career development fellowship that will enable her to continue the research of her PhD project and to apply for personal funding.

List of publications

Published

Prenatal stress-induced programming of genome-wide promoter DNA methylation in 5-HTT-deficient mice. Schraut KG, Jakob SB, **Weidner MT**, Schmitt AG, Scholz CJ, Strekalova T, El Hajj N, Eijssen LM, Domschke K, Reif A, Haaf T, Ortega G, Steinbusch HW, Lesch KP, Van den Hove DL. Transl Psychiatry. 2014 Oct 21;4:e473. doi: 10.1038/tp.2014.107.

Transl Psychiatry, Impact factor: 5.620 (2016)

5-HTT deficiency affects neuroplasticity and increases stress sensitivity resulting in altered spatial learning performance in the Morris water maze but not in the Barnes maze. Karabeg MM, Grauthoff S, Kollert SY, **Weidner M**, Heiming RS, Jansen F, Popp S, Kaiser S, Lesch KP, Sachser N, Schmitt AG, Lewejohann L. PLoS One. 2013 Oct 22;8(10):e78238. doi: 10.1371/journal.pone.0078238. eCollection 2013.

PLoSOne, Impact factor: 2.806 (2016)

Submitted

Differential anxiety-related behaviours and brain activation in Tph2-deficient female mice exposed to adverse early environment; Charlotte S Auth*; **Magdalena T Weidner***; Sandy Popp; Tatyana Strekalova; Angelika G Schmitt-Boehrer; Daniel LA van den Hove; Klaus-Peter Lesch*; Jonas Waider*

Epigenetic priming of a SOX10 binding site associated with the myelin basic protein (MBP) promoter by prenatal stress in mouse and man; **Magdalena Theodora Weidner**, MSc; Elise Beau Vangeel, PhD; Ehsan Pishva, PhD; Johanna Eva Maria Zoeller, MSc; Titia Hompes, Professor; Christiane Ziegler, PhD; Katharina Domschke, Professor; Klaus-Peter Lesch*, Professor; Stephan Claes*, Professor; Daniel Louis Albert van den Hove*, PhD

Impact of early-life stress on socio-emotional behaviour and the transcriptome and methylome within the amygdala of brain serotonin-deficient mice; **MT Weidner1**; R Lardenoije;L Eijssen; S Popp; R Palme; KU Förstner; T Strekalova; HWM Steinbusch; AG Schmitt-Boehrer; J Waider; DLA van den Hove*; KP Lesch*

In preparation

Differential susceptibility to prenatal stress exposure in serotonin transporter-deficient mice; an epigenetic perspective; **MT Weidner***; KG Schraut*; NK Leibold; Roy Lardenoije; L Eijssen; K Foerstner; T Strekalova; HWM Steinbusch; AG Schmitt-Boehrer; KP Lesch*; DLA van den Hove*

Gene-by-environment interaction in aggression: the role of developmental epigenetic programming; **MT Weidner**; J Waider; T Strekalova; KP Lesch*; DLA van den Hove*

List of figures

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List of abbreviations

Abbreviation	**Full name**
Chapter I	
5-HT	5-hydroxytryptamine, serotonin
5-HTT	serotonin transporter
5-HTTLPR	serotonin transporter gene-linked polymorphic region
GAD	generalised anxiety disorder
HPA	hypothalamo-pituitary-adrenal
MBP	myelin basic protein
MD	major depression
PTSD	posttraumatic stress disorder
TPH2	tryptophan hydroxylase 2
Chapter II	
5-HIAA	5-hydroxyindoleacetic acid
5hmC	5-hydroxymethylcytosine
5-HT	5-hydroxytryptamine, serotonin
5-HTP	5-hydroxytryptophan
5-HTR	serotonin receptor
5-HTT	serotonin transporter
5-HTTLPR	serotonin transporter gene-linked polymorphic region
5mC	5-methylcytosine
ADHD	attention deficit hyperactivity disorder
ADX	adrenalectomised
ADXr	ADX with GC replacement

AOB	accessory olfactory bulb
AR	androgen receptor
AVP	arginin vasopressin
AVPR1a and b	arginin vasopressin receptor 1a and b
Bdnf	brain-derived neurotrophic factor
BNST	bed nucleus of the stria terminalis
CMS	chronic mild stress
COMT	Catechol-O- Methyltransferase
CpG	cytosine-phosphate-guanine
CREB	cAMP-response element binding protein
CRH	corticotrophin releasing hormone
CRHR	corticotrophin releasing hormone receptor
CSF	cerebrospinal fluid
DNMT	DNA methyltransferase
DR	dorsal raphe
ESR1	estrogen receptor alpha
FC	frontal cortex
GC	glucocorticoid
GIRK2	G protein-gated inward rectifying potassium channel subunit-2
GR	glucocorticoid receptor
HDAC	histone deacetylase
HPA	hypothalamo-pituitary-adrenal
MAO-A	monoamine oxidase a
mCORT	metabolic corticosterone
MOE	main olfactory epithelium
MR	median raphe
MS	maternal separation
MTL	medial temporal lobe
NGFI-A	Nerve Growth Factor-Induced Protein A
OXT	oxitocin
OXTR	oxitocin receptor
PAG	periaqueductal grey
PFC	prefrontal cortex
PS	prenatal stress
PVN	paraventricular nucleus
Tfm	testicular feminisation model
TPH2	tryptophan hydroxylase2
VMAT	vesicular monoamine transporter
vmPFC	ventromedial prefrontal cortex
VNO	vomeronasal organ
WHO	world health organisation

Chapter III

5-HT	5-hydroxytryptamine, serotonin
5-HTT	serotonin transporter
ADHD	attention deficit hyperactivity disorder
ARG	alpha-fetoprotein related protein
Bm	base mean
CBP	cAMP-response-element-binding-protein-binding protein
Cck	cholecystokinin
Chr	chromosome
Clock	Clock circadian regulator
CRH	corticotrophin releasing hormone
DEG	differentially expressed gene
DLB	dark-light box
DMF	dfferentially methylated fragment
DR	dorsal raphe
EIA	enzyme immuno assay
EPM	elevated-plus maze
GO	gene ontology
GxE	gene-by-environment
HPA	hypothalamo-pituitary-adrenal
IL-5	interleukin 5
IZKF	interdisciplinary Centre for Clinical Research
lfcSE	log2 fold change standard error
lg2FC	log2 fold change
MAO-A	monomamine oxidase A
mCORT	metabolic corticosterine
MS	maternal separation
NGFI-A, EGR1	early growth response protein1, nerve-growth factor induced protein A
OF	open-field
PFC	prefrontal cortex
PFC	postnatal day
PVN	paraventricular nucleus
RIT	resident-intruder test
RQI	RNA quality indicator
RT	room temperature
S	supplementary results
SM	supplementary material
stat	Wald statistic
Tph2	tryptophan hydroxylase2
WT	wild type

Chapter IV

3-CST	3-chamber sociability test

5-HT	5-hydroxytryptamine, serotonin
5-HTT	serotonin transporter
5-HTTLPR	serotonin transporter gene-linked polymorphic region
AFM	Afamin
Bm	base mean
CBP	cAMP response element binding protein binding protein
ChIP	chromatin immunoprecipitation
Chr	chromosome
CLDN11	claudin11
CORT	corticosterone
CR	control redundancy
DEF	differentially enriched fragment
DEG	differentially expressed gene
DNMT	DNA methyltransferase
Dup	duplicates
Dusp1	dual specificity phosphatase1
E	embryonic day
EGR1	early growth response protein1
EPM	elevated-plus maze
FA2H	fatty acid 2-hydoxylase
Fabp7	fatty acid binding protein
FL	fragment length
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Gjb1	gap junction protein beta1
Gjc2	gap junction protein gamma2
GR	glucocorticoid receptor
Hapln2	hyaluronan and proteoglycan link protein 2
HPA	hypothalamo-pituitary-adrenal
IN	input control
IP	immunoprecipitated
IZKF	interdisciplinary Centre for Clinical Research
lfcSE	log2 fold change standard error
lg2FC	log2 fold change
M Seq	sequences in million
MAG	myelin associated glycoprotein
MBP	myelin basic protein
MYRF	Myelin Regulatory Factor
NGFI-A	berve growth factor induced protein A
Nxpe2	neurexophilin and PC-esterase domain family member 2
OL	oligodendrocyte
Olig1	oligodendrocyte transcription factor1
pCORT	plasma corticosterone

Per1	period circadian clock1
PS	prenatal stress
PS	postnatal day
PST	Porsolt swim test
qRT-PCR	quantitative real-time PCR
RES	resilient
RQI	RNA quality indicator
RT	room temperature
Sgk1	serum/glucocorticoid regulated kinase1
Slc6a9	solute carrier family 6 member 9
SOX10	Sex Determining Region Y-Box 10
stat	Wald statistic
SUS	susceptibility
Tph2	tryptophan hydroxylase2
TR	treatment redundancy
WT	wildtype

Chapter V

ρA	density
5-HT	5-hydroxytryptamine, serotonin
BL	basolateral amygdala
BSA	bovine serum albumine
CB	calbindin
Ce	central amygdala
DLB	dark-light box
DR	dorsal raphe
GAD	generalised anxiety disorder
HPA	hypothalamo-pituitary-adrenal
IEG	immediate early gene
IN	interneuron
La	lateral amygdala
MS	maternal separation
NHS	normal horse serum
OF	open-field
P	postnatal day
PAG	periaqueductal grey
PFA	paraformaldehyde
PN	principal neuron
PV	parvalbumin
PVN	paraventricular nucleus
RT	room temperature
TPH2	tryptophan hydroxylase

Chapter VI

3-CST	3-chamber sociability test
5-HTT	serotonin transporter
5-HTT	5-hydroxytrypatmine, serotonin
5-HTTLPR	serotonin transporter gene-linked polymorphic region
Arhgap26	Rho GTPase Activating Protein 26
AUC	area under the curve
biDNA	bisulfite DNA
CA	cornu ammonis
CBP	cAMP-response-element-binding-protein-binding protein
cDNA	copy DNA
CORT	corticosterone
DAB	3,3'-diaminobenzidine
DG	dentate gyrus
E	embryonic day
EDS	Edinburgh Depression Scale
EGR1	early growth response protein1
EPM	elevated-plus maze
GCL	granular cell layer
gDNA	genomic DNA
Hes5	hairy/enhancer of split 5
ir	immunoreactivity
Limch1	LIM and calponin homology domains1
MBP	myelin basic protein
NDS	normal donkey serum
NGS	normal goat serum
OL	oligodendrocyte
Olig	oligodendrocyte lineage transcription factor
orL	oriens layer
P	postnatal day
PFA	paraformaldehyde
PRAQ	pregnancy-related questionnaire
PS	prenatal stress
PST	Porsolt swim test
qRT-PCR	quantitative real-time PCR
RT	room temperature
Sox10	Sex Determining Region Y-Box 10
SSRI	selective serotonin reuptake inhibitor
STAI	Spielberger State Trait Anxiety Inventory

Chapter VII

5-HT	5-hydroxytryptamine, serotonin
5-HTT	5-HT transporter
BL	basolateral amygdala

CCK	cholecystokinin
Ce	central amygdala
CRH	corticotrophin releasing hormone
CRHR1	CRH receptor 1
DEF	differentially enriched fragment
DEG	differentially expressed gene
DLB	dark-light box
DMF	differentially methylated fragment
DOHAD	developmental origin of health and disease
DR	dorsal raphe
GAD	generalised anxiety disorder
La	lateral amygdala
MAO-A	monoamine oxidase A
MS	maternal separation
OF	open-field
Olig1	oligodendrocyte transcription factor 1
PS	prenatal stress
PTSD	posttraumatic stress disorder
PVN	paraventricular nucleus
TPH2	tryptophan hydroxylase2

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1 Publication (Chapter 5):
Differential anxiety-related behaviours and brain activation in Tph2-deficient female mice exposed to adverse early environment

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Under Review at **European Journal of Neuropsychopharmacology**

Participated in	**Author Initials,** Responsibility decreasing from left to right						
Study Design Methods Development	MTW	CSA	DvdH	JW	KPL	AGSB	TS
Data Collection	CSA	MTW					
Data Analysis and Interpretation	MTW	CSA	JW	DvdH	KPL	SP	
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	MTW MTW CSA MTW MTW	CSA CSA MTW CSA	 JW JW	 DvdH DvdH			

Explanations (if applicable):

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2 Publication (Chapter 6):
Epigenetic priming of a SOX10 binding site associated with the myelin basic protein (MBP) promoter by prenatal stress in mouse and man

Magdalena Theodora Weidner1,2, MSc; Elise Beau Vangeel3, PhD; Ehsan Pishva2,4, PhD; Johanna Eva Maria Zoeller1, MSc; Titia Hompes3,6, Professor; Christiane Ziegler5, PhD; Katharina Domschke5, Professor; Klaus-Peter Lesch1,2,7*, Professor; Stephan Claes3,6*, Professor; Daniel Louis Albert van den Hove1,2*, PhD

*equal contribution

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Rejected by **Translational Psychiatry**
Planned submission to **PLOSone**

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Study Design Methods Development	MTW	EV	SC	DvdH	KPL	TH	KD	CZ
Data Collection	MTW	EV	JZ	TH				
Data Analysis and Interpretation	MTW	EP	DvdH	KPL				
Manuscript Writing Writing of Introduction Writing of Materials & Methods Writing of Discussion Writing of First Draft	MTW MTW MTW MTW MTW MTW	DvdH DvdH DvdH 						

3 Publication (Chapter 3):
Impact of early-life stress on socio-emotional behaviour and the transcriptome and methylome within the amygdala of brain serotonin-deficient mice

MT Weidner1,2; R Lardenoije2; L Eijssen2,4; S Popp1; R Palme6; KU Förstner5; T Strekalova1,2,3; HWM Steinbusch2; AG Schmitt-Böhrer1; J Waider1; DLA van den Hove1,2*; KP Lesch1,2,3*

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Participated in	**Author Initials,** Responsibility decreasing from left to right							
Study Design Methods Development	MTW	DvdH	KPL	LE	TS	SP	AGSB	
Data Collection	MTW							
Data Analysis and Interpretation	MTW	DvdH	KPL	RL	LE	JW	KF	HWMS
Manuscript Writing	MTW	DvdH						
Writing of Introduction	MTW	DvdH						
Writing of Materials & Methods	MTW	DvdH						
Writing of Discussion	MTW	DvdH						
Writing of First Draft	MTW	DvdH						

4 Publication (Chapter 4):
Differential susceptibility to prenatal stress exposure in serotonin transporter-deficient mice; an epigenetic perspective

MT Weidner*1,2; KG Schraut1,3*; NK Leibold2; R Lardenoije2; L Eijssen2,4; K Förstner5; T Strekalova1,2,6; HWM Steinbusch2; AG Schmitt-Böhrer1; KP Lesch1,2,6*; DLA van den Hove1,2*

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Planned submission to **EMBO Journal**

Participated in	**Author Initials,** Responsibility decreasing from left to right							
Study Design Methods Development	KGS	DvdH	MTW	KPL	AGSB	TS		
Data Collection	KGS	MTW	NKL					
Data Analysis and Interpretation	MTW	KGS	DvdH	KPL	LE	RL	HWMS	KUF
Manuscript Writing	MTW		DvdH					
Writing of Introduction	MTW		DvdH					
Writing of Materials & Methods	KGS	MTW	DvdH					
Writing of Discussion	MTW	KGS	DvdH					
Writing of First Draft	KGS	MTW	DvdH					

Explanation (if applicable):

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Gene-by-environment interaction in aggression: the role of developmental epigenetic programming
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To be submitted to **Progress in Neurobiology**

Participated in	**Author Initials,** Responsibility decreasing from left to right							
Concept	MTW	KPL						
Manuscript Writing Writing of Introduction Writing of Discussion Writing of First Draft	 MTW MTW MTW							
Revision	DvdH	KPL	TS	JW				

It has to be mentioned that the titles displayed might not represent the final title of the manuscripts and that dependent on the revision Process the manuscripts might still be alternated so that the manuscripts in this thesis might not display the exact versions that will finally be published in a scientific Journal.

The doctoral researcher confirms that she/he has obtained permission from the co-authors for legal second publication.

The doctoral researcher and the primary supervisor confirm the correctness of the above mentioned assessment.

_____

Doctoral Researcher's Name Date Place Signature

_____

Primary Supervisor's Name Date Place Signature

“Dissertation Based on Several Manuscripts“

Statement of individual author contributions to figures/tables/chapters included in the manuscripts

Figures

1 Publication (Chapter 5):

Differential anxiety-related behaviours and brain activation in Tph2-deficient female mice exposed to adverse early environment

Charlotte S Auth1*; Magdalena T Weidner1,2*; Sandy Popp1; Tatyana Strekalova1,2,3; Angelika G Schmitt-Böhrer1; Daniel LA van den Hove1,2; Klaus-Peter Lesch1,2,3*; Jonas Waider1*

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1	MTW	CSA	DvdH	JW	KPL	AGSB	SP	TS
2	CSA	MTW	JW	DvdH	KPL	AGSB		
3	MTW	CSA	DvdH	JW	KPL	AGSB	SP	TS
4	CSA	MTW	JW	DvdH	KPL	AGSB		
5	CSA	MTW	JW	DvdH	KPL	AGSB		

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Figure	**Author Initials,** Responsibility decreasing from left to right							
1	MTW	EP	EV	DvdH	SC	TH	CZ	KD
2	MTW	EP	DvdH	JZ	KPL			

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Impact of early-life stress on socio-emotional behaviour and the transcriptome and methylome within the amygdala of brain serotonin-deficient mice

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Figure	**Author Initials,** Responsibility decreasing from left to right					
1	MTW	DvdH	KPL	SP	AGSB	HWMS
2	MTW	DvdH	KPL	JW	TS	
3	MTW	DvdH	KPL	JW	TS	
4	MTW	DvdH	KPL	LE	KUF	
5	MTW	DvdH	KPL	RL	KUF	

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MT Weidner1,2*; KG Schraut1,3*; NK Leibold2; Roy Lardenoije2; L Eijssen2,4; KU Förstner5; T Strekalova1,2,6; HWM Steinbusch2; AG Schmitt-Böhrer1; KP Lesch1,2,6*; DLA van den Hove1,2*

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Planned submission to **EMBO Journal**

Figure	**Author Initials,** Responsibility decreasing from left to right						
1	KGS	MTW	NKL	DvdH	KPL	AGSB	TS
2	KGS	MTW	NKL	DvdH	KPL	AGSB	TS
3	KGS	MTW	NKL	DvdH	KPL	AGSB	TS
4	KGS	MTW	KUF	DvdH	KPL	LE	
5	MTW	RL	DvdH	KPL	LE		

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To be submitted to **Progress in Neurobiology**

Figure	**Author Initials,** Responsibility decreasing from left to right							
1	MTW	KPL						
2	MTW	KPL	JW					
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4	MTW							

Tables

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I also confirm my primary supervisor's acceptance.

_____

Doctoral Researcher's Name Date Place Signature

Affidavit

I, hereby confirm that my thesis entitled "***Brain serotonin throughout development-for better and for worse***" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process, neither in identical nor in similar form.

Place, Date Signature

Hiermit erkläre ich an Eides Statt, die Dissertation „***Der Effekt von Serotonin im sich entwickelnden Gehirn-in guten wie in schlechten Tagen***" eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters angefertigt, und keine anderen, als von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher, noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum Unterschrift

Supplementary chapter

Work published in the supplementary chapter was conducted partially during the Master thesis. Even though this paper has been published under the lead of Dr. Schraut, my work contributed significantly to the results:

Prenatal stress-induced programming of genome-wide promoter DNA methylation in 5-HTT-deficient mice. Schraut KG, Jakob SB, **Weidner MT**, Schmitt AG, Scholz CJ, Strekalova T, El Hajj N, Eijssen LM, Domschke K, Reif A, Haaf T, Ortega G, Steinbusch HW, Lesch KP, Van den Hove DL. Transl Psychiatry. 2014 Oct 21;4:e473. doi: 10.1038/tp.2014.107.

Figure 2

Supplementary Figure 1

https://www.nature.com/articles/tp2014107

https://www.nature.com/articles/tp2014107#supplementary-information